IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellant: Donald L. Wise, Debra J. Trantolo, David D. Hile and Stephen A. Doherty

Serial No.: 10/613,975 Art Unit: 1645

Filed: July 3, 2003 Examiner: Khatol Shahnan-Shah

For: *VACCINES TO INDUCE MUCOSAL IMMUNITY*

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

This is an appeal from the final rejection of claims 1 and 3-11 in the Office Action mailed June 16, 2004, in the above-identified patent application. A petition to revive this application and fee, discussed below, and Notice of Appeal and fee, accompanies this Appeal Brief. The Commissioner is hereby authorized to charge $1000.00, including the fee for filing the Notice of Appeal and this Appeal Brief for a large entity, to Deposit Account No. 50-3129. This Appeal Brief is also accompanied by a Petition for Revival of an Application for Patent Abandoned Unintentionally Under 37 CFR 1.137(b) and the authorization to charge $1,500.00, the fee for filing this Petition for a large entity, to Deposit Account No. 50-3129. It is believed that no additional fee is required with this submission. However, should an additional fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

(1) REAL PARTY IN INTEREST

The real party in interest of this application is Depuy Mitek, a Johnson & Johnson company.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to appellant, the undersigned, or appellant's assignee which directly affects, which would be directly affected by, or which would have a bearing on the Board's decision in this appeal.

(3) STATUS OF CLAIMS

Claims 1 and 3-11 are pending. Claims 12-21 and 3 have been cancelled. Claims 1 and 3-11 are on appeal. The text of each claim on appeal, as pending, is set forth in an Appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

An amendment after final rejection was mailed on August 10, 2004. In the Advisory Action mailed November 3, 2004, the Examiner indicated that this amendment would be entered. An appendix sets forth the claims on appeal.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 1 defines a vaccine composition for inducing an immune response to a pathogen comprising a nucleic acid encoding an antigen eliciting an immune response to the pathogen encapsulated in a mucoadhesive controlled release particulate formulation comprising an open-celled polymeric foam of approximately 95% void volume, or particles thereof. Support for claim 1 can be found in the specification at least at page 8, lines 5-6, 16-18 and 20; at page

11, lines 1-2; page 20, lines 10-11; page 10, lines 26-27; page 25, lines 17-20 and claim 1 as originally filed. As defined by claim 3 the composition comprises a mucoadhesive polymer coating (see at least page 25, lines 17-20). As defined by claim 4, the composition comprises an enteric outer coating or capsule (see at least page 20, lines 28-31).

As defined by claim 5, the composition has a particulate diameter of less than five microns (see at least page 8, lines 12-13). Claim 8 defines the polymer as a low molecular weight poly(D,L-lactide-co-glycolide) (see at least page 8, lines 3-5). Claim 9 defines the pathogen as selected from the group consisting of malaria, tularemia, anthrax, and *Helicobacter pylori* (see page 7, lines 20-21). Claim 10 defines the composition as also containing an adjuvant (see at least page 23, lines 9-10). Claim 11 defines the antigen as expressed or released for a period of weeks to months (see at least page 8, lines 13-16).

As defined by claim 6 the composition is formed by lyophilizing a solution of a biodegradable polymer to form an open-celled polymeric foam of approximately 95% void volume (see at least page 8, lines 5-6), impregnating the foam with an aqueous solution of the nucleic acid (see at least page 8, lines 6-7, lyophilizing the foam to remove the water (see at least page 8, lines 7-8, and extruding the resulting matrix at ultrahigh pressures (see at least page 8, line 8). As defined by claim 7, the method also contains the step of cryogenically grinding the matrix to an average particle size of fifteen microns in diameter; and sieving to isolate particles less than five microns in diameter (see at least page 8, lines 10-13).

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues presented on appeal are:

(1) whether claims 1 and 3-11 are enabled as required by 35 U.S.C. § 112, first paragraph.

(7) GROUPING OF CLAIMS

The claims do not stand or fall together. Reasons for this grouping and arguments for the separate patentability of these groups of claims are provided below.

(8) ARGUMENT

(i) The Invention

Mucous membranes are the primary routes of entry for a large number and wide variety of disease carrying agents. Many pathogens enter and replicate at the mucosal surface before causing systemic infection. The mucosal immune system can be stimulated by oral administration. However, the induction of mucosal immunity has been shown to depend on a number of variables including the delivery system. Local administration of antigens usually requires large amounts of antigen to produce a response (see at least page 9). At page 9, the specification also states that delivery of antigen is key to developing an immune response and that under-stimulation may fail to prime the immune system. The present application relates to the development of effective and long-lasting vaccines, especially vaccines incorporating nucleic acid encoding antigen, such as plasmid DNA, by encapsulating the DNA within a mucoadhesive controlled release particulate formulation.

As discussed at least at page 17, administration of naked DNA leaves such vaccines vulnerable to attack by enzymes that can reduce the half-lives to minutes or hours. Chemical modification can increase the half life of the vaccines, but this may also increase systemic

toxicity. The Examiner argues that the claims are not enabled for all pathogens. However, appellants are not claiming any unique DNA, merely DNA encoding antigens that are present in pathogens. Vaccines, including DNA vaccines, have been widely available for a long time. The invention here is to put them into a mucoadhesive controlled release particulate formulation. As discussed at least at page 8, the mucoadhesive controlled release particulate formulation stimulates and maintains the immune response to pathogens. The application clearly provides support for such a formulation. See pages 17-28. In addition, the specification provides examples demonstrating the efficacy of these formulations.

(ii) Rejections under 35 U.S.C. § 112, first paragraph, enablement

Claims 1 and 3-11 were rejected as non-enabled for the breadth of pathogens encompassed by the claim language.

Claims 1, 3-8, 10 and 11 are drawn to a vaccine composition for inducing an immune response to a pathogen comprising a nucleic acid encoding an antigen eliciting an immune response to the pathogen encapsulated in a mucoadhesive controlled release particulate formulation comprising an open-celled polymeric foam of approximately 95% void volume, or particles thereof.

Claim 9 is limited to three specific pathogens: malaria, tularemia, anthrax, and *Helicobacter pylori*. The examiner has provided no rational as to why claim 9 is rejected since the claim is neither overly broad, being limited to four specific pathogens, and her remarks indicated that she believes the claims are enabled for "certain pathogens", although she fails to specify which pathogens.

The Legal Standard for Enablement

The Court of Appeals for the Federal Circuit (CAFC) has described the legal standard for enablement under § 112, first paragraph, as whether one skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation. See, e.g., *Amgen v. Hoechst Marion Roussell* 314 F.3d 1313 (Fed. Cir. 2003) and *Genentech, Inc. v. Novo Nordisk A/S*, 108 F3d at 165, 42 USPQ2d at 1004 (quoting *In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). See also *In re Fisher*, 427 F.2d at 839, 166 USPQ at 24; *United States v. Telectronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988); and *In re Stephens*, 529 F.2d 1343 (CCPA 1976). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *M.I.T. v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985). In addition, as affirmed by the Court in *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524 (Fed. Cir. 1987), a patent need not teach, and preferably omits, what is well known in the art.

Whether the disclosure is enabling is a legal conclusion based upon several underlying factual inquiries. See *In re Wands*, 858 F.2d 731, 735, 736-737, 8 USPQ2d 1400, 1402, 1404 (Fed. Cir.1988). As set forth in *Wands*, the factors to be considered in determining whether a claimed invention is enabled throughout its scope without undue experimentation include the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. In cases that involve unpredictable factors, "the scope of the enablement

obviously varies inversely with the degree of unpredictability of the factors involved." *In re Fisher*, 427.F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation ' must not be unduly extensive.' *In re Atlas Powder Co., v. E.I. DuPont De Nemours & Co.* , 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir.1984).

As noted in *Ex parte Jackson*, the test is not merely quantitative, since a considerable amount of experiment is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed. See *Ex parte Jackson*, 217 USPQ 804, 807 (PTO Bd. App. 1982). The adequacy of a specification's description is not necessarily defeated by the need for some experimentation to determine the properties of a claimed product. *See Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F3d 956, 965-966 63 USPQ2d 1609, 1614 (Fed. Cir. 2002). There is no requirement for examples.

As the Board of Patent Appeals recently quoted in another case,

"Nevertheless, "[w]hen rejecting a claim under the enablement requirement of section 112," it is well settled that "the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes, of course, providing sufficient reasons for doubting any assertions in

the specification as to the scope of enablement." *In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993)."

Analysis

The Examiner states that the specification, while being enabled for a composition inducing an immune response against certain pathogens, does not reasonably provide enablement for a vaccine inducing immune response against all pathogens. Unfortunately, there is no analysis as to what pathogens are enabled, which are not, nor why they are not. Instead, the remarks seem to go to the issue of whether or not DNA vaccines generally are enabled. However, the claims are drawn to an improved DNA vaccine formulation generally, not a specific vaccine. Appellants do not claim to have invented DNA vaccines, and indeed have provided much evidence to show that DNA vaccines are known. The specification and application instead are drawn to the advantages obtained using the polymeric carrier.

It appears that the Examiner has mischaracterized the present application. The present application is directed to compositions which provide controlled release of DNA vaccines. The claims define encapsulating nucleic acid encoding an antigen eliciting an immune response to a pathogen in a mucoadhesive controlled release particulate formulation. The invention here is to place the DNA into a mucoadhesive controlled release particulate formulation to achieve sustained delivery of the vaccine and to maintain an immune response.

The best evidence against the examiner's rejection is the article cited by the Examiner in the Office Action mailed December 22, 2003, O'Hagan, J. Pharm. Pharmacol. 50:1-10 (1997) ("O'Hagan"), a copy of which is enclosed in the Appendix, **dated four years before the**

priority date of this application. O'Hagan makes clear that even as of 1997, nucleic acid vaccines, while not being perfect and having some FDA issues, were effective and could be delivered using a polymeric carrier.

Additional papers were enclosed with the Amendment and Response filed August 10, 2004 to show that DNA vaccines are considered to be enabled and vaccination with them does not require "undue experimentation". See Pachuk, et al. Curr Opin Mol Ther. 2(2):188-98 (April 2000); Barnes, et al. Curr Opin Mol Ther. 2000 Feb;2(1):87-93 (February 2000); and Watts and Kennedy Int. J. Parasitol. 29(8):1149-63 (1999) ("Watts"), copies of which are enclosed in the Appendix.

The claims define a vaccine composition for inducing an immune response to a pathogen that contains a nucleic acid encoding an antigen eliciting an immune response to the pathogen encapsulated in a mucoadhesive controlled release particulate formulation. The composition can be formed by a method that contains the following steps: (1) lyophilizing a solution of a biodegradable polymer to form an open-celled polymeric foam of approximately 95% void volume, (2) impregnating the foam with an aqueous solution of the nucleic acid, (3) lyophilizing the foam to remove the water, and (4) extruding the resulting matrix at ultrahigh pressures.

Applying the *Wands* factors, it is clear from the amount of direction and guidance in the specification that sufficient detail is provided to one of ordinary skill in the art to make and use the claimed composition.

The quantity of experimentation, the state of the prior art, the relative skill of those in the art, and the predictability of the art

The skill of one in the art is high. A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984). The genetic manipulation of plasmid DNA is **highly** routine in the art. As described in Watts, plasmid vectors can be rapidly constructed and easily tested. All that is required is the antigen DNA sequence. Watts and the specification on pages 4-6 and pages 11-17, for example, disclose a number of DNA vaccines for bacterial, viral, and parasitic pathogens suitable for use in the claims of the present application. Therefore, the creation of numerous, different plasmids encoding antigens from a variety of pathogens would be routine experimentation for one of ordinary skill in the art. In addition, the skill of one in the art with respect to incorporation of active agents into polymers is also high (for example, see page 21, last paragraph). There is also predictability in the art with respect to delivery of vaccines by polymeric particles (for example, see page 20, lines 2-18).

The amount of direction and guidance presented, the presence of working examples, the nature of the invention

The claims define compositions wherein nucleic acid encoding an antigen is encapsulated in a mucoadhesive controlled release particulate formulation. The specification describes the use of these vaccine compositions to induce an immune response against pathogens such as malaria, anthrax, tularemia, and *H. pylori*. Appellants submit that these examples are only meant to

demonstrate the utility of the compositions and should not limit the scope of the claims. As discussed above, the specification at least at pages 4-6 and pages 11-17, disclose a number of DNA vaccines for bacterial, viral, and parasitic pathogens suitable for use in the claims of the present application. The specification discloses the encapsulation of DNA in a biodegradable polymer to achieve slow release into the system at least at pages 17-20. The specification discloses the addition of a mucoadhesive at least at pages 21-23. The specification at least at page 26 describes appropriate size ranges for the particles as defined by the claims. The specification discloses the formation of the vaccine composition by a method including the steps of lyophilizing a solution of a biodegradable polymer to form an open-celled polymeric foam of approximately 95% void volume, impregnating the foam with an aqueous solution of the nucleic acid, lyophilizing the foam to remove the water, and extruding the resulting matrix at ultrahigh pressures at least at pages 27-28. The specification discloses administration of the vaccine at least at page 32. While there is no requirement for examples, the specification provides *in vitro* data verifying antigen release at least at pages 29-31. Finally, the specification provides ***in vivo*** data in BALB/c mice immunized with vaccine/PLGA particles, PLGA-alone, or a control oligodeoxynucleotide/PLGA particles verifying protective immunity only in mice immunized with the vaccine/PLGA particles (see pages 32-33).

As discussed above, the prior art teaches the use of DNA in the production of vaccines against a vast array of diseases (see Watts and Kennedy Int. J. Parasitol. 29(8):1149-63 (1999), a copy of which is enclosed in the Appendix). It is clear from the discussion above that the specification discloses the claimed composition in such detail that one skilled in the art would be

able to make and/or use the invention without undue experimentation. Thus the claims are enabled by the specification.

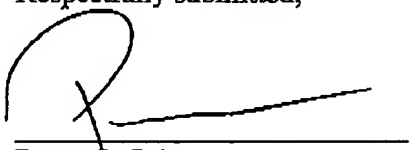
(9) SUMMARY AND CONCLUSION

The claims are drawn to vaccine compositions incorporating nucleic acid encoding antigen, such as plasmid DNA, made by encapsulating the DNA within a mucoadhesive controlled release particulate formulation. The standard for enablement is whether one skilled in the art would be able to make a vaccine as claimed. The prior art and specification teach the use of DNA in the production of vaccines against a vast array of diseases. One could easily substitute these DNA sequences into the vaccine compositions described in the current application to induce an immune response. Appellants are not claiming any unique DNA, merely DNA encoding antigens that are present in pathogens when incorporated into a mucoadhesive controlled release particulate formulation. The application clearly provides support for such a formulation, and provides actual working examples.

As also discussed above, claim 9 is not drawn to a broad range of pathogens, but to four specific pathogens. It appears in generalizing the rejection to all of the independent claims that the examiner has failed to individually examine the dependent claims, as required.

For the foregoing reasons, Appellant submits that claims 1 and 3-11 are enabled.

Respectfully submitted,



Patrea L. Pabst
Reg. No. 31,284

Date: February 22, 2006

PABST PATENT GROUP LLP
400 Colony Square, Suite 1200
1201 Peachtree Street
Atlanta, Georgia 30361
(404) 879-2151
(404) 879-2160 (Facsimile)

Claims Appendix: Claims On Appeal

1. A vaccine composition for inducing an immune response to a pathogen comprising a nucleic acid encoding an antigen eliciting an immune response to the pathogen encapsulated in a mucoadhesive controlled release particulate formulation comprising an open-celled polymeric foam of approximately 95% void volume, or particles thereof.

3. The composition of claim 1 further comprising a mucoadhesive polymer coating.

4. The composition of claim 1 further comprising an enteric outer coating or capsule.

5. The composition of claim 1 having a particulate diameter of less than five microns.

6. The composition of claim 1 formed by

lyophilizing a solution of a biodegradable polymer to form an open-celled polymeric foam of approximately 95% void volume,

impregnating the foam with an aqueous solution of the nucleic acid,

lyophilizing the foam to remove the water, and

extruding the resulting matrix at ultrahigh pressures.

7. The composition of claim 1 wherein the method further comprises cryogenically grinding the matrix to an average particle size of fifteen microns in diameter; and

sieving to isolate particles less than five microns in diameter.

8. The composition of claim 1 wherein the polymer is a low molecular weight poly(D,L-lactide-co-glycolide).

9. The composition of claim 1 wherein the pathogen is selected from the group consisting of malaria, tularemia, anthrax, and *Helicobacter pylori* .

10. The composition of claim 1 further comprising providing an adjuvant with the antigen.

11. The composition of claim 1 wherein the antigen is expressed or released for a period of weeks to months.

Evidence Appendix

1. O'Hagan, J. Pharm. Pharmacol. 50:1-10 (1997).

2. Pachuk, et al. Curr Opin Mol Ther. 2(2):188-98 (April 2000).

3. Barnes, et al. Curr Opin Mol Ther. 2000 Feb;2(1):87-93 (February 2000).

4. Watts and Kennedy Int. J. Parasitol. 29(8):1149-63 (1999).

Related Proceedings Appendix

None.

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J. Pharm. Pharmacol. 1997, 49: 1–10
Received September 15, 1997

Recent Advances in Vaccine Adjuvants for Systemic and Mucosal Administration*

DEREK T. O'HAGAN

Chiron Corporation, 4560 Horton Street, Emeryville, CA 947608, USA

Abstract

Although vaccines produced by recombinant DNA technology are safer than traditional vaccines, which are based on attenuated or inactivated bacteria or viruses, they are often poorly immunogenic. Therefore, adjuvants are often required to enhance the immunogenicity of these vaccines. A number of adjuvants which are particulates of defined dimensions ($< 5\ \mu m$) have been shown to be effective in enhancing the immunogenicity of weak antigens in animal models. Two novel adjuvants which possess significant potential for the development of new vaccines include an oil-in-water microemulsion (MF59) and polymeric microparticles. MF59 has been shown to be a potent and safe adjuvant in human subjects with several vaccines (for example HSV-2, HIV-1 and influenza virus). An MF59 adjuvanted influenza has been recommended for approval in Italy.

Microparticles prepared from the biodegradable polymers the poly(lactide-co-glycolides) (PLG) are currently undergoing extensive pre-clinical evaluation as vaccine adjuvants. Because of their controlled release characteristics, microparticles also possess considerable potential for the development of single dose vaccines. The development of single dose vaccines would offer significant advantages and would improve vaccination uptake rates in at risk populations, particularly in the developing world. In addition to systemic administration, microparticles have also also been shown to enhance the immunogenicity of vaccines when administered by mucosal routes. Therefore microparticles may allow the development of novel vaccines which can be administered by non-parenteral routes. Mucosal administration of vaccines would significantly improve patient compliance by allowing immunization to be achieved without the use of needles.

An alternative approach to the development of mucosally administered vaccines involves the production of genetically detoxified toxins. Heat labile enterotoxin (LT) from *Escherichia coli* and cholera toxin from *Vibrio cholerae* are two closely related bacterially produced toxins, which are the most potent adjuvants available. However, these molecules are too toxic to be used in the development of human vaccines. Nevertheless, these toxins have been modified by site-directed mutagenesis to produce molecules which are adjuvant active, but non-toxic. The most advanced of these molecules (LTK63), which has a single amino acid substitution in the enzymatically active subunit of LT, is active as an adjuvant, but non-toxic in pre-clinical models. The approach of genetically detoxifying bacterial toxins to produce novel adjuvants offers significant potential for the future development of mucosally administered vaccines.

The widespread use of vaccines, which can be defined as agents that induce protective immunity against a pathogen, has had a significant impact on man's health for more than a hundred years. With the exception of the provision of clean water supplies, vaccines represent the most cost-effective public health intervention strategy and their use results in the prevention of many millions of deaths a year (Plotkin & Mortimer 1988; Levine et al 1997). The most notable vaccine success stories are the global eradication of smallpox in 1977, the planned eradication of poliomyelitis by the year 2000 and the Expanded Programme on Immunization (EPI). In the early 1970s, fewer than 5% of the world's infants were immunized against routine childhood infections, resulting in millions of deaths a year from vaccine-preventable diseases. In 1974, the World Health Organization and other interna-

*Conference Science Medal 1997 lecture presented at the British Pharmaceutical Conference, Scarborough, September 15–18, 1997.

Table 1. The characteristics of an 'ideal' vaccine: the Children's Vaccine Initiative. New York. 1990.

Effective after a single-dose
Can be administered early in life
Administered by a mucosal route, preferably orally
Heat-stable during transport and storage
Affordable throughout the world
Applicable to a wide range of diseases

tional agencies initiated the EPI and by 1990, global infant immunization coverage had risen to 80%. More recently, in 1990, the Children's Vaccine Initiative (CVI) was established in New York. Within the CVI, the characteristics of an 'ideal' vaccine for future development were defined (Table 1). Although the goal of developing such a vaccine is a daunting task, significant steps have already been made towards this objective (Levine et al 1997).

New Strategies in Vaccine Development

Vaccines have traditionally consisted of live attenuated pathogens, whole inactivated organisms or inactivated toxins (Table 2). Despite the successes of traditional approaches to vaccine development, alternative approaches are attractive or necessary for a number of reasons, particularly those related to safety. Some live attenuated vaccines can cause disease in immunosuppressed individuals, or in the general population, at very low incidence, through reversion to a more virulent phenotype. Whole inactivated vaccines (e.g. *Bordetella pertussis* and influenza virus) contain reactogenic components which can cause undesirable side-effects in some individuals. Furthermore, some pathogens (e.g. hepatitis B, hepatitis C, human papillomavirus and *Plasmodium* spp) are difficult or even impossible to grow in culture, restricting the opportunity to develop vaccines by traditional methods.

In the last decade several new approaches to vaccine development have emerged which might solve some of the problems associated with traditional vaccines. The approaches include:

- recombinant sub-unit vaccines based on proteins produced in mammalian cells, yeast, bacteria or baculovirus;
- synthetic peptides representing important epitopes from pathogens;
- conjugate vaccines, based on bacterial polysaccharides conjugated to carrier proteins; and
- DNA vaccines, in which genes encoding antigens from pathogens are administered directly.

These new approaches have already resulted in the development of several new vaccines (Table 3).

Table 2. Examples of the traditional approaches to vaccine development.

	Viral	Bacterial
Live, attenuated pathogens	Measles Vaccinia	Mycobacterium BCG *Salmonella typhi*
Inactivated pathogens	Rabies Hepatitis A	*Bordetella pertussis* *Vibrio cholerae*
Toxoids	Not applicable	Tetanus toxoid Diphtheria toxoid
Combination vaccines	MMR	DPT

MMR measles, mumps and rubella vaccine; DPT, diptheria, tetanus and pertussis (whole cell) vaccine.

The first recombinant sub-unit vaccine was developed after the cloning and expression of hepatitis B surface antigen into yeast cells by two of the founders of Chiron Corporation (Valenzuela et al 1982). More recently, the first rationally designed vaccine was developed at Chiron, using site-directed mutagenesis to render the toxin from *B. pertussis* immunogenic but non-toxic (Pizza et al 1989). Polysaccharide-protein conjugate vaccines were first introduced world-wide in the early 1990s against *Haemophilus influenzae* type b, the principal cause of bacterial meningitis in young children. Bacterial polysaccharides are poorly immunogenic, particularly in young children, because they are not recognized by T helper cells. Therefore, the polysaccharides were chemically cross-linked to a protein carrier molecule (usually diphtheria or tetanus toxoid) which was recognized by T cells. These vaccines have proven to be tremendously successful, eliminating Hib disease in some countries and causing a decline in the disease of greater than 95% in others, depending on the immunization level in the population at risk (Rothbrock et al 1995). This success has motivated the development of conjugate vaccines against meningococcus A and C and against pneumococcus. New approaches to vaccine development, in combination with recent advances in adjuvants and delivery systems, have resulted in an expansion of the potential markets for vaccine products and in the establishment of new market areas (Table 4).

DNA immunization

It was discovered in the early 1990s that direct intramuscular injection of plasmid DNA encoding an antigen resulted in the induction of antibody and cell-mediated immune responses and protective immunity (Donnelly et al 1997). The use of DNA

Table 3. Recent successes in vaccine development.

Hepatitis B vaccine	Recombinant hepatitis B surface antigen expressed in yeast or mammalian cells (Valenzuela et al 1982)
Acellular pertussis vaccine	Including a genetically detoxified pertussis toxin (Pizza et al 1989)
Polysaccharide-conjugate vaccines	Bacterial polysaccharides from *Haemophilus influenzae* type b conjugated to a protein carrier (normally tetanus or diphtheria toxoids). Similar vaccines for Meningococcus A and C and Pneumococcus are in development

for vaccination has the potential to revolutionize vaccine development. Plasmid-based DNA vaccines would be very stable and easy and inexpensive to produce. In addition, they would enable the inclusion of many antigens simultaneously, should work even in the presence of maternal antibodies and should induce potent cytotoxic T-cell responses, because antigens produced within host cells should have access to the major histocompat, cability (MHC) class I antigen presentation pathway. DNA immunization is likely to be the safest approach to the development of vaccines against dangerous pathogens (e.g. Ebola virus), because researchers would not be required to work with the pathogen directly. Because antigens encoded by DNA are expressed by host cells, this approach might be optimally exploited to vaccinate against viruses or intracellular bacteria, particularly if attenuated vaccines are not considered an acceptable approach because of safety concerns (e.g. HIV).

However, DNA vaccination remains largely unproven in large-animal models, including man, and there are significant safety concerns which need to be addressed. The potential for genomic integration of DNA and the possibility of insertional mutagenesis is a concern, as too is the induction of an immune response against DNA and the potential for the subsequent generation of autoimmunity. Moreover, in studies performed so far in small-animal models, DNA vaccines have not out-performed alternative approaches, involving immunization with recombinant proteins or attenuated organisms (Manickan et al 1997). Therefore, it seems prudent in the short-term to focus the use of DNA vaccines on to pathogens for which no vaccines are currently available and for which alternative approaches have not proven successful (e.g. HIV, HSV, herpes complex virus (HCV), malaria and *Chlamydia trachomatis*).

Table 4. Potential market growth areas for vaccine development.

Adolescent vaccines	e.g. HIV, HSV, HPV, aP.
Vaccines for the elderly	e.g. Influenza, VZV.
Travellers vaccines	e.g. ETEC, *V. cholerae*, *S. typhi*, Shigella.
Combination vaccines	e.g. DTaP/Hib, DTaP/Hib/IPV.
Mucosal delivery systems	e.g. microparticles, LT/CT mutants.

HSV, herpes simplex virus; HPV, human papilloma virus; aP, acellular pertussis, VZV, Varicella zoster virus. ETEC, entertoxigenic *E. coli*, DTap, diptheria, tetanus and acellular pertussis-combined vaccine, IPV, inactivated polio virus. LT/CT, heat labile enterotoxin/cholera toxin.

New Directions in Vaccine Development: Vaccines as Drugs

An area with significant potential for vaccine development involves the use of vaccines as therapeutic agents, or 'drugs'. Rather than preventing disease, therapeutic vaccines (or immunotherapeutics) would be designed to eliminate or ameliorate existing diseases, including chronic infectious diseases. The main disease situations in which therapeutic vaccines might prove useful include:

- chronic infections e.g. those caused by HSV, HIV, HCV, hepatitis B vaccine (HBV), HPV or *Helicobacter pylori*;
- tumour vaccines e.g. melanoma, breast or colon cancer; and
- vaccines for allergy and autoimmunity e.g. multiple sclerosis, Type I diabetes and rheumatoid arthritis.

Preliminary studies have indicated that the potency of novel adjuvants (e.g. MF59 emulsion), might promote the development of therapeutic vaccines against chronic infectious diseases (Traquina et al 1996). Moreover, there has been renewed optimism that advances in our understanding of the complexities of the immune response might lead to the development of more effective immunotherapeutic approaches for the control and elimination of tumours (Williams 1996). In addition, studies in pre-clinical animal models have indicated that oral administration of antigens responsible for autoimmune diseases can result in amelioration of the disease process (Weiner 1997). DNA vaccines might have a significant role to play in the development of therapeutic vaccines. It has been suggested that the greatest potential for DNA vaccines is in the re-adjustment of the immune response when the 'normal'

response is inappropriate or ineffective (Manickan et al 1997).

Although the potential development of therapeutic vaccines offers exciting market opportunities, no therapeutic vaccine has yet proven sufficiently effective in a phase III clinical trial to justify the award of a licence. Future developments in this area of vaccinology are very much dependent on our ability to control and manipulate the immune response to a greater extent than has so far been achieved. However, selective manipulation of the immune response might be achieved through the development of novel adjuvants and delivery systems.

The Role of Adjuvants in Vaccine Development

Although newer approaches to vaccine development, particularly the use of recombinant proteins, offer significant advantages over more traditional approaches, a general problem is that the newer generation vaccines are often poorly immunogenic. This is mainly because these vaccines are more highly purified than traditional vaccines and, therefore, do not contain extraneous bacterial or viral components, which often function as built-in adjuvants. These residual components within vaccines e.g. the many proteins administered in the whole-cell *B. pertussis* vaccines, are often the main cause of reactogenicity.

Adjuvants were originally described by Ramon (1924) as "substances used in combination with a specific antigen that produced more immunity than the antigen alone" and this definition remains valid. Despite considerable research over many years, the only adjuvants currently approved for use with vaccines by the Federal Drug Administration of the USA are aluminium compounds (generically called alum). Alum has an excellent safety record, but comparative studies show that it is a relatively weak adjuvant for antibody induction and a poor adjuvant for the induction of cell-mediated immunity (Gupta et al 1995). Therefore, there is an urgent need for the development of new and improved adjuvants and delivery systems which are potent and safe and can be used with new generation vaccines.

The characteristics of an ideal adjuvant

The successful development of adjuvants requires consideration of a number of issues: the characteristics of an 'ideal' vaccine adjuvant are shown in Table 5. The most important issue is safety, because safety concerns have restricted the widespread use of adjuvants in man since alum was first introduced more than 50 years ago (Gupta et al 1995). Many experimental adjuvants have high potency, but are too toxic for clinical use. Adverse events associated with vaccine adjuvants can be a direct consequence of the inclusion of toxic or non-degradable components, or the inclusion of agents that over-activate the immune or inflammatory systems. For standard prophylactic immunization in healthy people, only those vaccine adjuvants that induce a minimum number of side-effects will be generally acceptable. However, for therapeutic vaccination against chronic viral, bacterial, neoplastic or autoimmune diseases more significant side-effects might be acceptable. Overall, an acceptable balance between vaccine potency and vaccine associated side-effects will need to be established in the clinic for each new vaccine application.

Additional issues important for adjuvant development include stability, ease of manufacture, cost, and applicability to a wide range of vaccines. A new adjuvant should have a shelf-life of at least a year, ideally at room temperature. The materials and processes used for adjuvant production will be subject to standard pharmaceutical constraints and suitably pure components should be available in sufficient quantity. Moreover, an ideal adjuvant for world-wide distribution should not add significant cost to the manufacture of a vaccine and should be capable of being administered with a range of antigens by a variety of different routes, including oral or intranasal.

Recent developments in vaccine adjuvants

The two adjuvants which have generated the most data in animal models are alum and Freund's

Table 5. The characteristics of an 'ideal' vaccine adjuvant.

Biodegradable and biocompatible
Should not be toxic, carcinogenic, teratogenic or abortogenic
Non-antigenic and not immunologically cross-reactive with tissue antigens
Induces a minimum of injection site reactogenicity
Simple well defined chemical structure
Induces a minimum of non-specific effects on the immune system
Acceptable for administration to man
Safe to administer to young and immunocompromised individuals
Effective for peptide, protein, polysaccharides and DNA
Effective after a single-dose
Induces both humoral and cell-mediated immunity
Capable of being administered orally
Induces systemic and mucosal immunity
Promotes antigen uptake by lymphoid tissues
Stable formulation which is inexpensive to manufacture
Can be manufactured reproducibly on a large scale
Good shelf-life, preferably without refrigeration
Easy to mix with antigen or combination of antigens

adjuvants. Freund's adjuvants are water-in-oil emulsions, which are generally more potent than alum. Freund's complete adjuvant contains immunostimulatory compounds from *Mycobacterium tuberculosis*, while the less toxic incomplete Freund's adjuvant contains only mineral oil and the emulsifier Arlacel A. Although incomplete Freund's adjuvant has been widely used in man, it has never been approved for commercial use despite the absence of significant toxicity (Gupta et al 1993). Alum is widely used in vaccines for man, but is a relatively poor adjuvant for most antigens and does not induce cell-mediated immunity (Gupta et al 1995). A simplified representation of the immune response to a vaccine antigen is shown in Figure 1.

The adjuvant activity of the immunostimulatory peptidoglycan from *M. tuberculosis* in Freund's complete adjuvant has been shown to reside in an *N*-acetyl muramyl-L-alanyl-D-isoglutamine (MDP) fraction. However, MDP also has significant toxicity. Nevertheless, a number of derivatives of MDP with reduced toxicity have been described and several have been evaluated in clinical trials (Ott et al 1992). A second class of immunostimulatory compounds which have been evaluated as adjuvants are derived from the lipopolysaccharide of Gram-negative bacteria. The most extensively evaluated member of this family, monophosphoryl lipid A, obtained from *Salmonella minnesota*, has been defined as penta- and hexaacyl derivatives of diglucosamine monophosphate (Ulrich & Ulrich 1995). Monophosphoryl lipid A has been evaluated in the clinic with a range of antigens and shown to have adjuvant activity, with tolerable side-effects (Ulrich & Ulrich 1995). A third group of immunostimulatory compounds which have been widely evaluated are the triterpenoid glycosides derived from *Quillaja saponaria*. The fraction QS21 was isolated by Kensil, who defined the structural groups responsible for adjuvant activity (Soltysik et al 1995). Clinical testing has shown some adjuvant activity for QS21, but also 'flu-like' side-effects (Livingston et al 1994).

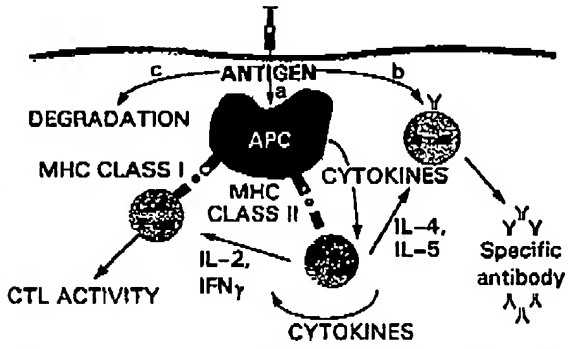

Figure 1. A simplified representation of the immune response to a vaccine antigen. Antigen injected intramuscularly might be: (a) taken up by antigen-presenting cells (APC); (b) bound to surface antibody on B cells; or (c) degraded. In general, adjuvants act to upregulate the immunological activity of any of the cell-types depicted. Antigen-delivery systems modify the distribution of antigen (or adjuvant) through these pathways. Antigen taken up by antigen-presenting cells is processed into peptide epitopes, which use two discreet pathways to MHC molecules (class I and II), which present peptide for interaction with either $CD8^+$ or $CD4^+$ T cells, respectively. The stimulated T cells might generate cytokine signals to upregulate the immune response or act as cytotoxic T lymphocytes. Antibodies are produced by B cells with help provided by the cytokines (interleukins (IL) and interferons (IFN) produced by the $CD4^+$ T cells.

The agents which the immune system has evolved to combat (viruses, bacteria and parasites) are particulate in nature and are normally efficiently phagocytosed by macrophages and other antigen-presenting cells. Therefore, it seemed rational to develop particulate antigen delivery systems (e.g. microparticles, iscoms, liposomes and emulsions) which can be manufactured in a size-range comparable with that of natural pathogens (20 nm to 2 μm).

The development of MF59, a submicron emulsion adjuvant

Despite the potency of Freund's adjuvants, unacceptable toxicity prompted researchers to pursue alternative approaches to the development of adjuvants. Liposomes have been evaluated extensively as vehicles for both antigens and adjuvants (Gregoriadis 1990; Alving 1992), but no liposomal vaccines have yet been commercially developed. Immunostimulatory fractions from *Quillaja saponaria* have been incorporated into particulate structures which contain antigen (iscoms) or are co-administered with free antigen (iscomatrix) (Bengtsson & Sjölander 1996). A successful vaccine against equine influenza has been developed using the iscoms approach and a vaccine for man is currently under clinical evaluation. The submicron squalene-water emulsion (MF59) was initially designed as a delivery vehicle for a synthetic MDP derivative, sodium *N*-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanyl-2-(1′, 2′-dipalmitayl-sn-glycero-3′phospho) ethylamide (MTP) (Ott et al 1995). The chemical components of MF59, the oil squalene (a terpenoid cholesterol precursor) and the surfactants sorbitan trioleate (span 85) and polyoxyethylene sorbitan mono-oleate (Tween 80) have all been used in medicinal products for man. In a number of animal models MF59/MTP was a significantly more potent adjuvant than alum and in some studies approached the potency of Freund's. However, no significant toxicity was observed with

MF59/MTP (Ott et al 1995). Rather surprisingly, further studies showed that the MTP was not necessary for the induction of potent antibody responses and that the emulsion alone was a potent adjuvant. Moreover, reduction of the size of the emulsion droplets from 1–2 μm to 200–300 nm resulted in a significant increase in adjuvant activity. Preclinical evaluation of MF59 resulted in the induction of antibody titres in a number of animal models, including primates, which were 5–50-fold higher than those achieved with alum (Ott et al 1995). The occurrence of unacceptable side-effects after administration of MTP to individuals previously exposed to HSV or influenza (Couch 1993) and the observation of the adjuvant effect of the emulsion alone, resulted in clinical evaluation of MF59 as a vaccine adjuvant.

Experience in the clinic (> 8000 subjects immunized), with vaccines including HIV, HSV, CMV, HBV and influenza, has shown that MF59 is safe and well tolerated in both seropositive and seronegative individuals (Kahn et al 1994; Langenberg et al 1995). Local effects were limited to transient erythema at the injection site and systemic effects were limited to flu-like symptoms, such as headache and fever, which tended to resolve within two days (Langenberg et al 1995). These effects were judged to be sufficiently minor to allow the vaccination of newborn infants with MF59 in a HIV vaccine trial. The potency of MF59 for HSV and HIV vaccines in phase I/II clinical trials has been shown to be comparable with preclinical models (Kahn et al 1994; Langenberg et al 1995). In the HSV clinical trial, the antibody titres induced by recombinant glycoproteins were significantly higher than those induced by the same antigens in combination with alum. Moreover, the neutralizing titres were similar to those induced after natural infection with the virus. In both the HIV and the HSV trials, strong helper T-cell responses were observed in seronegative individuals. The overall conclusion from all clinical trials with MF59 is that the adjuvant is safe and effective in man in combination with a variety of antigens. In a recent preclinical study in primates the range of antigens for which MF59 is effective was extended to include polysaccharide-protein conjugate vaccines (Granoff et al 1997).

Biodegradable microparticles as vaccine adjuvants

Over the last twenty years, the adjuvant effect achieved through the association of antigens with polymeric microparticles has been repeatedly demonstrated (O'Hagan 1994, 1997). Encapsulation of antigens into microparticles, including submicron particles, promotes their entry into lymph nodes and provides a high local concentration of antigen over an extended time-period. Microparticles also promote the interaction of encapsulated antigens with antigen-presenting cells e.g. macrophages.

The poly(lactide-co-glycolides), biodegradable and biocompatible polyesters, are primary candidates for the development of microparticles as vaccines, because they have been used in man for many years as suture material and as controlled-release delivery systems for peptide drugs (Wise et al 1979). However, the adjuvant effect achieved by the encapsulation of antigens into poly(lactide-co-glycolide) microparticles has been demonstrated only relatively recently (O'Hagan et al 1991a, b; Eldridge et al 1991). Particle-size was shown to be an important factor affecting immunogenicity, because smaller microparticles (< 10 μm) were significantly more immunogenic than larger particles (> 10 μm) (Eldridge et al 1991; O'Hagan et al 1993). The adjuvant effect of microparticles can also be enhanced by co-administration with additional adjuvants (O'Hagan et al 1991b). Recent studies have shown that microparticles also exert an adjuvant effect for cell-mediated immunity, including the induction of cytotoxic T-cell responses after both systemic and mucosal administration (Maloy et al 1994; Moore et al 1995). The induction of cytotoxic T-cell responses are important for the eradication of virally infected cells and for immune responses against alternative intracellular pathogens.

In the long-term one of the most attractive features of microparticles for vaccine development is their use to control the rate of release of entrapped antigens (O'Hagan 1997). Ultimately, this might enable the development of single-dose vaccines, through the preparation of microparticles which release entrapped antigens at the times when booster doses of vaccines would normally be administered. The development of a single-dose vaccine would represent a significant step towards the development of an ideal vaccine (Table 1) and would result in improved vaccine compliance, particularly in the developing world. In a recent study with rats a single immunization with tetanus toxoid entrapped in controlled-release microparticles induced immunity comparable with that after three doses of tetanus toxoid adsorbed on alum (Singh et al 1997). In addition, a single-dose of microparticles with an entrapped peptide (O'Hagan et al 1995) or protein (Cleland et al 1994) from HIV-1 induced neutralizing antibodies for at least one year. Nevertheless, further research is needed to promote the stability of antigens during microencapsulation and after in-vivo adminis-

Table 6. The advantages of poly(lactide-co-glycolide) microparticles for vaccine development.

Safety: biodegradable and biocompatible polymers
Acceptable for administration to man
Controlled-release might enable the development of single-dose vaccines
Adjuvants might be entrapped in the microparticles
Many antigens can be entrapped simultaneously in the micro particles
Microparticles might be administered by mucosal routes, including oral delivery
Antigens are protected from degradation in the intestine
Antigens are targeted to lymphoid tissue
Microparticles induce serum and secretory antibodies
Microparticles induce cell-mediated immunity
Freeze-dried formulations, with enhanced stability for entrapped antigens
Large scale manufacture of microparticles has already been achieved

tration. The potential advantages of microparticles for vaccine development are shown in Table 6.

Future developments in vaccine adjuvants: mucosal delivery

Mucosal administration of vaccines is an attractive approach which offers several significant advantages over the traditional approach to vaccine delivery, intramuscular injection. The advantages of mucosal delivery include easier administration, reduced side-effects and the potential for frequent boosting without the need for trained personnel. Moreover, mucosal delivery of vaccines is the only effective mean of inducing immune responses in the mucosal secretions of the body. This is important, because the majority of pathogens initially infect hosts through the mucosal tissues of the gut or the respiratory or genital tracts. In addition, because the protective barrier of the skin is not breached during mucosal administration, the potential for the introduction of infection through the use of 'dirty' needles is eliminated.

In mice, oral immunization with poly(lactide-co-glycolide) microparticles induced potent serum IgG, secretory IgA and systemic cytotoxic T-cell responses (Eldridge et al 1990; Challacombe et al 1992: Maloy et al 1994). Although relatively large doses of antigens were used in these studies (at least 100 μg), a single oral dose of 10 μg fimbriae from *B. pertussis* in microparticles protected mice from intranasal challenge (Jones et al 1996). In addition, intranasal immunization with 1–10 μg *B. pertussis* antigens in microparticles also induced protective immunity in mice against aerosol challenge (Cahill et al 1995; Shahin et al 1995). In primates, intra-tracheal or oral delivery of microencapsulated inactivated SIV in parenterally primed animals induced protective immunity against intra-vaginal challenge with the virus; systemic immunization alone did not protect (Marx et al 1993). Also in a primate study, intra-tracheal immunization induced protection against aerosol challenge with staphylococcal enterotoxin B (Tseng et al 1995). Recently, microparticles have also been shown to be effective for the oral delivery of plasmid DNA in mice (Matiowitz et al 1997).

Initial observations in small-animal models have indicated that rectal immunization might also be exploited using particulate antigen delivery systems (Zhou et al 1995). Delivery via the rectal route targets antigens to the abundant lymphoid tissues present in the local mucosal epithelium and avoids exposure to the enzymes and low pH of the upper gastrointestinal tract. Nevertheless, there are considerable problems associated with rectal delivery, including lack of cultural acceptability in some areas. In addition, the dosage form containing the vaccine might be expelled from the rectum before it has time to be effective and this might be difficult to control, particularly with young children.

Several different approaches to the mucosal delivery of vaccines have recently been evaluated, particularly for the oral route. These include live genetically attenuated bacterial vectors, including Salmonella and Lactococci spp, and live viral vectors, e.g. adeno, polio and vaccinia. In addition, non-living delivery systems have also been evaluated for the mucosal delivery of vaccines, including the toxins of *Vibrio cholerae* and *Escherichia coli*, lectins, liposomes and iscoms. These alternative approaches to mucosal vaccine delivery have recently been reviewed in multi-authored books (O'Hagan 1994; Kiyono et al 1996). A novel approach to the development of mucosal adjuvants is represented by the genetic manipulation of bacterial toxins, e.g. those of *V. cholerae* and *E. coli*, to render them non-toxic but still adjuvant-active (Douce et al 1995, 1997). The genetic manipulation of bacterial toxins was pioneered by Rappuoli's group (Pizza et al 1989), and was first applied to *B. pertussis* toxin for the development of a rationally designed, genetically engineered vaccine (Rappuoli 1997). Subsequently, the same approach has been applied to *V. cholerae* and *E. coli* toxins. A single amino acid substitution in the enzymatically active A sub-unit of *E. coli* toxin enabled the development of a completely non-toxic molecule (LTK63), which retained adjuvant activity when administered by several mucosal routes (Di Tommaso et al 1996). Native *V. cholerae* and *E. coli* toxins had previously been shown to be potent adjuvants after oral and intranasal administration to man, but they are too toxic to be used in vaccines. The modification of these

potent toxins to reduce or eliminate toxicity might enable their use as adjuvants for a wide range of vaccines. to be administered by several mucosal routes.

The prospects for the development of novel adjuvants

The successful use of MF59 in the clinic. and the absence of significant adverse effects suggests that several MF59-adjuvanted vaccines are likely to be developed in the near future. Indeed, the first product incorporating MF59 as an adjuvant, an influenza vaccine, was introduced on to the market in Italy in 1997. It is confidently expected that additional vaccine products incorporating MF59 as an adjuvant will be introduced in the coming years.

Although microparticles offer considerable promise for the development of new and improved vaccines, much work still needs to be done. The most important issue in relation to the potential development of single-dose vaccines is the stability of antigens in microparticles. Recent work has demonstrated that human growth hormone is stable in-vivo for at least one month after administration in microparticles (Johnson et al 1996). Nevertheless, additional work is needed to obtain a better understanding of the mechanisms governing protein stability in microparticles and the steps which can be taken to overcome specific problems with antigens.

A possible limitation of the use of microparticles as oral vaccines is their apparently low efficiency of uptake across the intestinal epithelium (O'Hagan 1996). Hence, it might prove attractive to target microparticles to the cells which are responsible for their uptake, the M cells of the Peyer's patches (Kato & Owen 1994). Traditionally, the oral route has proven difficult to exploit for vaccines, because of enzymatic degradation, dilution effects in the intestine, the low pH, and the poor absorption of proteins and peptides. Nevertheless, the oral route remains the most attractive approach and research will continue in this area. If oral delivery of microparticles proves too difficult to exploit clinically, intranasal delivery of microparticles might be an attractive option. The nasal cavity is readily accessible for administration and presents fewer problems than the gut. Moreover, many important pathogens initially infect the host through the nasal cavity. It seems likely that genetically detoxified toxins, e.g. those of *V. cholerae* and *E. coli*, will play an important role in the future development of intranasal vaccines. However, these molecules are likely to require formulation into specialized delivery systems to optimize their efficacy in the nasal cavity of man. Developments in oral delivery systems to protect mutants of the toxins of *V. cholerae* and *E. coli* against low pH and enzymatic degradation might also enable the development of orally administered vaccines.

It seems likely that future success in the mucosal delivery of vaccines will be driven by partnerships between those generating novel biological approaches and those able to deliver these molecules optimally to the relevant anatomical sites. Hence, it should be clear that pharmaceutical scientists have a valuable role to play in the future development of optimum delivery systems for both systemic and mucosal vaccines.

Conclusions

The only adjuvants currently approved for administration to man are aluminium compounds, which are weak adjuvants for antibody induction and are ineffective for the induction of cell-mediated immunity. In contrast, MF59 has been shown to be a potent and effective adjuvant in more than 8000 subjects in clinical trials, without inducing significant adverse effects. Hence, MF59 shows considerable promise for approval as a new vaccine adjuvant for a number of vaccines for man. In addition, microparticles represent an alternative approach to vaccine delivery which might have some advantages over MF59. Microparticles might prove particularly advantageous for mucosal delivery of vaccines or for the induction of cell-mediated immunity. In addition, microparticles might enable the development of single-dose vaccines through the use of controlled-release technology. Genetically detoxified bacterial toxins (e.g. LTK63) also show considerable promise for the future development of mucosally administered vaccines. However, the efficacy of microparticles and mutant toxins has yet to be demonstrated in man.

Through the advent of genomics and rapid screening techniques, the discovery of new antigens from pathogens has become quicker and more routine than ever before. Consequently, perhaps the greatest current challenge for vaccine development lies in the delivery of antigens for the induction of the optimum immune responses. This might be achieved through the use of novel adjuvants and delivery systems and might involve the selective induction of a specific antibody isotype or a T-cell subset, or it might involve the induction of mucosal immunity. Pharmaceutical scientists have much to contribute to the formulation of delivery systems for mucosal administration and to the selective targeting of antigens and adjuvants to specific antigen-presenting cells after systemic administration.

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DNA vaccines - challenges in delivery

Catherine J Pachuk[1*], Daniel E McCallus[1], David B Weiner[2] & C Satishchandran[1]

Addresses
[1]Wyeth-Lederle Vaccines
One Great Valley Parkway
Malvern
PA 19355
USA
Email: pachukc@war.wyeth.com

[2]505, Stellar-Chance Laboratories
Department of Pathology
University of Pennsylvania
422 Curie Boulevard
Philadelphia
PA 19104
USA

*Corresponding author

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DNA vaccines are typically comprised of plasmid DNA molecules that encode an antigen(s) derived from a pathogen or tumor cell. Following introduction into a vaccinee, cells take up the DNA, where expression and immune presentation of the encoded antigen(s) takes place. DNA can be introduced by viral or bacterial vectors or through uptake of 'naked' or complexed DNA.

Vaccination with DNA is a recent technology possessing distinct advantages over traditional vaccines (killed or attenuated pathogens) and the more recently developed subunit vaccines. Unlike most subunit vaccines, DNA vaccines induce both the humoral and cellular arms of the immune response. The stimulation of both arms of the immune system is important not only for the prevention of many diseases including AIDS, but also allows the use of a vaccine for therapeutic purposes. While the traditional attenuated pathogen vaccines are also able to elicit both cellular and humoral immune responses, there is a risk of reversion from the attenuated state to the virulent state. This risk does not exist with DNA vaccines. DNA vaccines can be manufactured and formulated by generic processes.

DNA vaccine technology, however, is still in its infancy and much research needs to be done to improve the efficiency with which these vaccines work in humans. While continued efforts toward improving both DNA expression and DNA delivery are equally important for increasing the utility of DNA vaccines, this review will focus both on non-viral delivery of plasmid DNA and delivery methods for the encoded antigen.

Keywords DNA condensation, DNA delivery, DNA vaccines, intracellular trafficking, lipoplex, transfection

Introduction

Unlike conventional drugs, plasmid DNA molecules are macromolecular and take part in complex interactions with cellular and extracellular biomolecules. Pharmacological activity of these prodrugs is dependent on their ability to be targeted to appropriate cell types, to be transported across the cell membrane, to have some degree of nuclease resistance and to maintain steady-state concentrations in the appropriate intracellular compartments. The polar and the anionic nature of plasmid molecules do not readily allow transfer across biological membranes [1]. Research into the delivery of nucleic acids has primarily focused on the delivery of antisense, triplex forming and ribozyme oligonucleotides, wherein the desired pharmacological properties have been developed into the structure without sacrificing the ability of oligonucleotide DNA and RNA molecules to function in base-pairing and catalysis. However, these developments cannot be readily translated to the delivery of plasmid DNA molecules, as they do not preserve the DNA molecule's ability to be expressed within the host cell. Therefore, the delivery of plasmid molecules has utilized either 'naked' DNA or agents that interact non-covalently with plasmid molecules.

In order for DNA vaccines to function, the cells of the vaccinee must internalize the DNA component of the vaccine. Although 'naked' DNA can be taken up by cells *in vivo*, the efficiency of the process is poor. Approximately 10^6 DNA molecules are taken up by approximately as many cells following the intramuscular inoculation of > 10^{11} molecules [2•]. Furthermore, the internalized DNA must then be transported to the cell nucleus such that the encoded antigen gene(s) can be transcribed and translated by the host cell enzymes. As both the processes of DNA internalization and nuclear localization are inefficient, the development of novel DNA delivery systems will be needed before DNA vaccines can realize their full potential. Structure-based rational designs for DNA delivery are limited. In addition, it is unclear as to which cell types should be targeted for DNA delivery for the optimal elicitation of immune responses. Therefore, current strategies are based both on rational design and empirical analysis [3]. It is the scope of this review to discuss current research and thinking in the field. Efforts to develop passive and active, and targeted and non-targeted plasmid DNA delivery systems will be described.

The route of DNA administration plays a fundamental role in DNA delivery. By altering the route of DNA administration, the magnitude and type of immune response can be modulated [4,5]. The modulation is thought to be effected through: (i) delivery of DNA to and subsequent transfection of different cell types; (ii) a difference in transfection efficiency at different sites; and/or (iii) differences in the transfected cell environment. A summary of the data available from these studies is presented.

Altering antigen delivery can also modulate immune response. There has been much innovation in the design of the DNA vaccine encoded antigen(s), with attempts to increase MHC class I and/or MHC class II presentation. Strategies have focused on both the intercellular and intracellular delivery of antigen to antigen-presenting cells (APCs) and to various antigen-processing pathways within a cell. Much has been learned from studies involving antigen delivery and these will be reviewed.

The success of DNA vaccines may require engineering of the immune response, possibly through the use of cytokines and costimulatory molecules. Inviting relevant cell types to participate in the uptake and processing of the antigen molecules is predicted to be relevant to the successful development of DNA vaccines. Literature in this area is reviewed with particular emphasis on conceptualization of the approaches and on the future of such approaches towards successful vaccine development.

DNA condensation

DNA condensation is a compaction process by which the inter-atomic distances are minimized between adjacent nucleotides. Condensation of DNA is a prerequisite to the uptake of DNA by cells, as condensed DNA occupies less space, there are more molecules per unit of space and there are fewer size-related restrictions on cell uptake. Although the major resistance to condensation is electrostatic repulsion, resistance is also contributed to by other unfavorable free energies. The relative contributions of these unfavorable free energies vary by several orders of magnitude [6,7••]. In supercoiled DNA (a topological state induced by two interwinding DNA strands where the number of interwindings is greater than the number of helical turns in the duplex) the free energy barriers are temporarily overcome for a given subset of sequences in a DNA molecule. The resultant torsional forces are transmitted into other parts of the molecule to relieve torsional stress, resulting in single stranded loops within the supercoiled molecule. Therefore, the supercoiled topological form is actually in equilibrium with other variant topological forms within a given DNA molecule.

The potent electrostatic barriers to DNA condensation can be overcome through the use of cations. Charge neutralization of DNA by cations relieves electrostatic repulsion, allowing DNA condensation to occur [8,9]. This free energy change causes a molecular collapse of plasmid DNA resulting in a 5- to 6-fold decrease in the hydrodynamic radii of the plasmid molecules, with a concomitant 2- to 3-orders of magnitude decrease in volume occupancy [10,11]. Condensation of DNA achieved through the use of cationic lipids, cationic microspheres, proteins and peptides will be discussed in the following sections. In addition to condensation, complexation with certain condensing agents confer other biophysical properties to the complexed DNA and to the surface architecture of the transfecting molecule.

Lipoplexes

Cationic lipids and their role in the complexation of DNA into lipoplex formation

Cationic lipids and cationic amphiphiles are ion-pair reagents that have both lipophilic and hydrophilic properties. Solubility of the lipid in aqueous solution is dependent upon the structure of specific hydrophilic and lipophilic groups. Depending upon the hydrophobic composition of the molecule, cationic lipids and amphiphiles can adopt a number of different liposomal structures. In polar solvents, a single chain lipid with a cationic headgroup will assemble into micelles containing hydrophobic cores, while certain lipids containing two or more hydrophobic chains can form bilayered vesicles [12]. A more complex liposomal structure is formed when a co-lipid or DNA is added. The multi-lipid interactions that occur through the addition of co-lipids bring about local rigidity in the otherwise fluid environment of mono-lipid liposomes, which further increases the asymmetry of the complex, causing a reassortment of bilayers (if present) and the possible creation of bilayers where originally there were none. Particles with distinctly different biophysical properties from the mono-lipid liposomes are thereby generated. Differences in these properties may impact the ability of these particles to be taken up by cells [13]. In addition, the presence of some neutral co-lipids such as dioleoyiphosphatidylethanolamine (DOPE) or cholesterol appear to increase transfection capabilities through processes hypothesized to invoke endosomal evasion or escape following cellular uptake [14].

Addition of DNA to cationic liposomes results in the charge neutralization of both the cationic lipids and DNA. Both the charge neutralization of DNA and the reordering of DNA on the cationic headgroups result in condensation of the DNA molecule. A concomitant reordering of the lipids in the liposomes also occurs upon addition of DNA. Unless controlled assembly processes are used, the resultant lipoplex particles are heterogeneous in both size and structure.

Lipoplexes as a facilitator of in vitro transfection

Complexes of DNA and lipids made by adding DNA to preformed cationic liposomes (lipoplexes), have been found to greatly facilitate the transfection of DNA into tissue cultured cells. Transfection of cells by lipoplexes was originally thought to occur by fusion of the lipoplex with cellular membranes. In fact, the observed transfer of phosphatidyl lipids from lipoplexes into cellular membranes was taken as proof for lipoplex/cell membrane fusion. Several laboratories have shown, however, that liposomes do not generally fuse with cellular membranes, rather the uptake is through a passive phagocytic or endocytic process [15,16]. Entry of DNA appears to be facilitated through increased residence time near an actively phagocytic cell. It has also been suggested that the presence of lipoplexes in the vicinity of a cell may in fact induce phagocytosis and or endocytosis to occur in that cell.

Aggregation of lipoplex particles has been observed to occur during cell transfection procedures. Transfection of cells is induced by, or occurs with, particles of a certain size range only; other sized particles are refractory to or actually inhibit this process. In order to increase the efficiency of DNA delivery to cells, it will be important to further elucidate the biochemical and biophysical properties of the transfecting particles. It will also be necessary to develop controlled assembly processes, such as those described by Gregoriadis and Szoka [17,18], in order to reproducibly generate lipoplex particles that are homogeneous in both size and structure.

Lipoplexes and the immune response

DNA immunization using non-viral vectors requires efficient methods for the delivery of plasmid DNA to cells *in vivo*. Although lipoplexes made by adding plasmid DNA to preformed cationic liposomes have been successful in

enhancing transfection in cell culture, these cationic lipid-DNA complexes, in general, do not result in increased transfection after *in vivo* intramuscular injection. In fact, these complexes appear to inhibit both the expression of the complexed DNA and the generation of subsequent immune responses when compared to the use of DNA alone [19•,20].

Dehydrated-rehydrated vesicles (DRVs) [17,21••], comprised of phosphatidylcholine (PC), DOPE, cationic lipids and DNA, enhance immune responses relative to those responses elicited by DNA alone when administered intramuscularly [21••] or subcutaneously [22]. Although these vesicles can be of a similar chemical composition to the traditionally made lipoplexes, DRV particles are smaller and more uniform in size. In addition, recent data suggests that the basic architecture of DRVs is different from that of traditionally made lipoplexes. Using anion competition, DNA was displaced from the traditionally formed lipoplexes but not from DRVs, suggesting that the DNA in lipoplexes is externally bound but is internalized in DRVs [23]. DNA may be similarly competed from lipoplexes by negatively charged proteins *in vivo*, perhaps explaining the failure of these complexes as DNA delivery vehicles for DNA vaccination. Although protection of DNA from nucleases has previously been cited as a proof of DNA entrapment within traditionally made lipoplexes [24], the premise of these experiments is questionable since DNA complexed with cationic lipids may not be a nuclease substrate. The true substrate is DNA coordinated to a divalent cation through the oxygen atom of the phosphoryl group.

As DRVs (but not traditionally formed lipoplexes) function in the intramuscular and subcutaneous delivery of DNA, it seems likely that the biophysical properties as well as the chemical composition of cationic lipid DNA complexes must be taken into consideration for the development of DNA delivery vehicles.

Local anesthetics and their role in DNA vaccine technology

Although naked DNA can transfect muscle cells *in vivo*, pretreatment of muscle with the local anesthetic bupivacaine several days prior to injection of DNA, results in increased DNA uptake, as evidenced by increased DNA expression at the injection site [25,26]. Increased expression of DNA is also associated with increased immune responses to antigens encoded by the injected DNA [27]. Increase in muscle transfection by pretreatment with bupivacaine is thought to be due to the myogenic activity of bupivacaine. Treatment of muscle with bupivacaine causes muscle fiber degeneration and subsequent recruitment of myoblasts into muscle regeneration [25]. Dividing myoblasts are postulated to be more likely to be transfected with DNA than the non-dividing, differentiated muscle cells, and are presumably responsible for the observed increase in muscle cell transfection. In addition, the recruitment of inflammatory cells to the site of bupivacaine injection may also allow for transfection of immune cells [25,26,28,29]. Due to the temporal nature of muscle cell degeneration and regeneration, the window of opportunity to transfect regenerating muscle fibers is 1 to 7 days post-bupivacaine treatment.

It has been shown that mixtures of DNA and bupivacaine delivered simultaneously also result in increased transfection and expression in muscle cells. The mechanism of muscle cell transfection observed for simultaneous delivery of DNA and bupivacaine is not related to the mechanisms involved with bupivacaine pretreatment [Pachuk CJ, Ciccarelli RB, Samuel M, Bayer M, Troutman R, Zurawski D, Schauer JI, Sosnoski DM, Higgins TI, Weiner DB, Zurawski V Jr, Satishchandran C, manuscript in preparation]. Interactions between bupivacaine and DNA have also been discovered that may explain the observed increase of *in vivo* transfection when bupivacaine and DNA are administered simultaneously.

Microspheres and cochleates

Microspheres are biodegradable particles designed for slow drug release. Adsorbed or entrapped DNA has been successfully delivered, following intramuscular injection, to APCs using microsphere particles comprised of PLG (poly(lactide-co-glycolide)) [30-32]. Efficiency of uptake by APCs is dependent upon microsphere size. As microspheres afford considerable protection to entrapped DNA, they are potential DNA delivery vehicles for mucosal and oral routes of immunization. In fact, oral DNA vaccination with microspheres has been shown to induce both mucosal and systemic immune responses to the encoded HIV envelope antigen [33].

Microspheres coated with cationic molecules to which the DNA is then adsorbed, allow for higher DNA expression following *in vivo* cellular uptake when compared to 'naked' DNA [34]. Intramuscular administration of these cationic microparticles also increases the immune response to the encoded antigen by 3-orders of magnitude, relative to the 'naked' DNA control [34].

Cochleates are 'jelly-roll' like structures formed by the bridging of neutral lipids in liposomes [35]. This bridging is mediated by divalent cations such as Ca^{2+}. Cochleates are stable in biological fluids and have been successfully used to deliver protein antigens.

Plasmid DNA can also be entrapped into cochleates and delivered to cells *in vivo* [36]. Due to the exclusion of water during cochleate formation, entrapped plasmid DNA molecules are predicted to be stable to acid environments, and hence these particles may be of use for oral DNA vaccine administration. Future research will determine the viability of this approach for the delivery of plasmid-based vaccines.

Targeted and active transfection processes for *in vivo* DNA delivery

Cellular uptake and nuclear localization of DNA follow the rules of mass action. Maximum bioavailability of DNA can only be realized through active transfection processes, which can be achieved through either physical means or through the use of targeted DNA delivery systems.

Physical methods of DNA delivery include biolistic injection, electroporation and iontophoresis. Although these processes afford rapid uptake of injected DNA, transfection

of DNA is limited to the surfaces and areas that are accessible to the devices themselves. In some studies, gene gun technology, which utilizes biolistic injection, has been found to elicit immune responses of much higher magnitude than those elicited through the use of DNA alone [37].

Targeted DNA delivery systems are biochemical approaches involving the use of ligands that interact with and bind to cell surfaces. These ligands can be complexed with DNA through cationic molecules [38,39] or through oligonucleotides that form triplex strands with DNA. DNA can be targeted to specific cell types through the use of a ligand or an antibody specific for that cell type [40,41•,42]. Alternatively, DNA can be actively transfected into any cell type by targeting receptors present on all cell types.

Conferring targeting capabilities to the DNA is predicted to result in only modest increases in *in vivo* delivery. Following the initial cell surface interactions, the DNA delivery vehicle should be designed to be able to either fuse with the cell membrane or to participate in an active cellular uptake mechanism. Use of efficient cell attachment and entry mechanisms derived from viral systems, such as Sendai virus, have resulted in 10-fold more efficient liposome uptake compared to those liposomes lacking this additional viral feature [43,44].

A problem associated with ligand-mediated DNA delivery systems, is that the internalized complex becomes localized within the endosomal compartment. However, the presence of membrane disrupting agents within the complex can allow escape from the endosome [38,39]. Internalized DNA must also be translocated to the nucleus where it can be expressed. Current research has focused on the use of nuclear localization signals that can be linked to the DNA through the use of either peptide nucleic acids (PNAs) that form triplex structures with the plasmid DNA molecule or modified lysines [45,46•].

Although targeted delivery systems are the most desired methods for DNA delivery, the methods to achieve them are still unclear. Knowledge of the desired cell types to target and which cell types to avoid are primary requirements for the successful development of these systems. As our knowledge continues to grow, it is not unrealistic to conclude that non-viral gene delivery systems will reach the efficiency and specificity of viral-based delivery systems.

Routes of administration

In order to modulate the level and type of immune response, plasmid DNA has been administered to human and animal vaccinees through a number of different routes and by a number of different methods. Until the actual mechanisms of immune stimulation effected by DNA vaccines are determined, the optimal site and/or method of administration may need to be determined on a case-by-case basis. For example, different types of immune response eliminate various pathogenic microorganisms. Knowledge gained regarding the precise means by which immune effectors are stimulated following each method and route of DNA administration will allow researchers and clinicians to direct particular DNA vaccines in a way which stimulates the immune response in a particular manner.

Intramuscular immunization

Intramuscular (im) injections are often used to administer DNA vaccines, frequently leading to a robust immune response. Uptake of plasmid and expression by myocytes in the area of injection has been demonstrated [47••]. It is doubtful, however, that myocytes are acting as APCs due to low expression of essential immune costimulatory molecules, such as B7-2 [48]. The unlikely possibility of myocytes acting as APCs has been eliminated by studies using MHC-chimeric mice in which bone marrow-derived cells were shown to be the actual APCs responsible for immunity following DNA vaccine administration [49,50••,51]. Dendritic cells (or possibly macrophages [52]) have been proposed as the primary APCs following im injection [53], although it is not yet clear if these cells were directly transfected with plasmid DNA or had taken up protein produced by transfected myocytes (a phenomenon known as crosspriming [54•]). One study involving a DNA vaccine encoding non-secreted protein has suggested, however, that direct expression of protein inside APCs may be necessary for the generation of immunity following im injection [55].

The stimulation of antibody production following DNA vaccination may be occurring through similar mechanisms. Antibodies are usually generated following the ingestion of foreign (exogenously produced) protein by APCs, which then digests these proteins to peptides for subsequent presentation to the immune system. This would indicate that cells transfected with plasmid DNA must express the protein and transfer it to classical APCs. However, non-secreted proteins have been shown to induce some levels of antibody production following DNA immunization [56]. These proteins may perhaps be released from the cell by apoptosis or may be secreted via a non-classical pathway.

Cutaneous immunization

The presence of a large number of resident APCs (Langerhans and dendritic cells) in the skin makes this area an advantageous site of immunization. Intradermal (id) immunization with DNA vaccines has proven to be an effective method of generating a specific immune response. In fact, topical application of DNA to the skin leads to a measurable immune response to the encoded antigen [57]. Coating of plasmid DNA onto gold beads and delivering them into cells with a 'gene gun' was more effective in eliciting an immune response when compared to DNA given im, intranasally (in) or intravenously (iv) [58]. A number of studies have shown that cells at the site of immunization are necessary for the induction of the immune response; both migratory and non-migratory cells are probably important [59-62].

It has been observed, in general, that gene gun-immunization typically leads to a Th2 response, while delivery of plasmid via needle injection induces a Th1 response [63]. Exceptions to this 'rule' have been noted [64] and the type of response generated may be, at least partially, antigen-dependent. The larger quantities of DNA needed for im injection may non-specifically push the immune response in a Th1 direction because of immune stimulatory sequences (ISS) present on plasmid DNA [65]. A Th1 response can be elicited following gene gun immunization if plasmids containing the genes for *interleukin-12* or *interferon-α* are codelivered [66].

Mucosal Immunization

An effective induction of mucosal immunity would be advantageous in preventing disease, as mucosal sites are the points of entry into the body for a variety of pathogenic bacteria and viruses. Selective enhancement of secretory IgA (sIgA) and specific cytotoxic T-lymphocytes (CTLs) in regional lymph nodes has been attempted through in, oral and intravaginal delivery of DNA vaccines. To prevent degradation of the DNA, it has been necessary to form complexes between the DNA and various (usually lipidic) substances. Expression of the appropriate protein has been observed in lungs [58], nasal tissue [67], spleen and draining lymph nodes [68] following in administration of DNA vaccines. Intranasal administration of plasmid containing the genes for HIV *env* and *rev* lead to increases in specific CTLs in the spleen and regional lymph nodes [69]. While in administration of DNA alone usually results in a Th2 response [69], this can be skewed in the Th1 direction by complexing with various lipidic substances [68,70] or by co-administration of plasmids containing the genes for the appropriate cytokines [69].

The goal of an oral DNA vaccine is complicated by the need to protect the DNA during its journey through the stomach. Microencapsulation of plasmid DNA into particles containing PLG lead to both specific mucosal IgA and serum antibodies [30]. Intestinal IgA and specific serum antibodies for rotavirus VP6 antigen, as well as some degree of protection from challenge, were observed following oral administration of PLG-encapsulated plasmids containing the *VP6* gene [71]. Oral delivery of plasmids in other formulations have also led to specific mucosal immune responses [72].

Specific antibodies (IgG and IgA) were found in vaginal washes following intravaginal administration of plasmids containing the HIV *env* and *rev* genes [73]. Similar results were found when rats were immunized intravaginally with the gene for human growth hormone [74]. Intravaginal immunization of a pregnant chimpanzee with genes from HIV led to specific IgA and IgG in the saliva and sera but not in vaginal washes [75].

While a great deal of research continues in the area of mucosal DNA immunization, it is clear that much remains to be learned and that administration of DNA through multiple sites may be necessary to achieve protective immunity.

Presentation of antigen

Intercellular and intracellular targeting of antigen can be accomplished through simple changes in antigen design. Rerouting of antigens via these changes modulates both the level and the type of response, presumably by increasing MHC class I and or MHC class II presentation.

MHC class I

The intracellular production of antigen following the administration of DNA vaccines is the hallmark of DNA vaccination. Intracellular antigen synthesis allows MHC class I presentation of antigen-derived peptides that can prime cellular immune responses. The generation of peptides for MHC class I presentation is, in part, mediated through the ubiquitination of endogenously produced proteins. Ubiquitination occurs when the C-terminal glycine of ubiquitin forms an amide isopeptide linkage with the ε-amino group of one or more exposed lysine residues of a substrate protein. Once linked to the substrate protein, ubiquitin itself becomes a target for ubiquitination, resulting in the polyubiquitination of the substrate protein. The polyubiquitinated substrate protein is then degraded by the 26S proteosome complex. A subset of the degradation products is comprised of peptides that can associate with MHC class I molecules.

The magnitude of a cellular response may be heightened by increasing the efficiency with which an antigen is targeted for ubiqitination. Our knowledge of ubiquitination signals is limited however, and there appear to be multiple signals. In addition, ubiquitination is catalyzed by a myriad of ubiquitin carrier proteins that are specific for certain proteins [76•]. As the expression of these carrier proteins is also regulated according to cell type and cell cycle, it seems unlikely that there is a universal system for ubiquitination of all proteins in all cell types, at all points in the cell cycle. Nonetheless, there have been some reported successes at targeting DNA vaccine-encoded antigens into the ubiquitin pathway. Rodriguez *et al* have demonstrated enhanced CTL induction in mice immunized with DNA constructs encoding an N-terminal ubiquitin fusion protein [77,78]. Enhanced responses were seen when ubiquitin was fused to either a viral gene or minigene. The ubiquitin moiety of these fusion proteins is thought to be a substrate for ubiquitination, thereby targeting the entire fusion protein to the proteosome complex. This would require that a lysine residue become exposed in the ubiquitin moiety. As folding of the ubiquitin moiety is expected to be modulated by the downstream fusion partner, exposure of a lysine residue is not predicted to occur in all fusion proteins. The universality of this strategy will undoubtedly be tested over the next few years.

Another strategy for attempting to increase cellular immune responses has involved the removal of signal peptides from antigens destined for secretion. This forces the cytosolic localization and the increased intracellular accumulation of antigen proteins, which may allow for increased MHC class I presentation of antigen peptides. Initial data has shown that mice immunized with plasmids expressing cytosolic localized HSV-2 gD antigen have higher lymphoproliferative responses to gD than mice immunized with plasmids expressing secreted or membrane-associated gD [Higgins TJ, Herold KM, Arnold RL, McElhiney SP, Pachuk C, unpublished data]. Boyle *et al* have demonstrated higher CTL responses in mice immunized id with a DNA vaccine expressing cytosolic localized antigen [79]. Interestingly, when immunized im, higher CTL responses were seen in those mice receiving constructs expressing secreted or membrane-associated antigen. It is not clear why this apparent discrepancy exists.

In addition to accessing the MHC class I processing pathway by an endogenous route, virus-like particles (VLPs) and some proteins appear to also access this pathway exogenously [80,81]. Following secretion or export from cells, these VLPs

and proteins gain entry to the cytosol of other cells (perhaps by binding to a cell surface receptor), where they gain access to the MHC class I processing pathway. Therefore, for a subset of antigens, cytosolic localization and or proteosome targeting may not be necessary for the elicitation of optimal cellular immune responses.

MHC class II

The intracellular production of antigen that occurs following DNA vaccination, results in a population of antigen molecules that become localized within APCs. It is unclear whether the antigen found in APCs merely reflects antigen taken up by an APC or whether some antigen is actually synthesized in an APC; most likely it is combination of these two events. Localization of antigen within an APC allows for the MHC class II presentation of antigenic peptides. The production of these peptides is achieved by antigen degradation in a lysosome-dependent pathway.

There has been much innovation in the design of DNA vaccines in an attempt to enable more efficient class II presentation of antigen. These modifications have included alterations to the design of the antigen molecule, expression of accessory molecules and changes in regulatory elements such as the promoter.

In general, changes to the antigen are designed to result in the increased availability of antigen to APCs. These changes most often involve modifications that allow the antigen to be exported out of the cell. Comparisons of immune responses following DNA vaccination with plasmids expressing secreted or cell-associated antigen, have demonstrated that it is possible to increase the magnitude of the immune response by targeting the antigen for secretion. For example, both Geissler and Inchauspe have reported higher seroconversion rates and higher antibody titers in BALB/c mice immunized with plasmid constructs expressing various forms of secreted hepatitis C (HCV) core protein, as compared with those mice immunized with constructs expressing cell-associated core [82,83]. Secretion of bovine herpesvirus 1 gD (84) and HSV-2 gD has also been shown to induce higher antibody titers in mice relative to those mice receiving constructs encoding cell-associated forms of the antigen [Higgins TJ, Herold KM, Arnold RL, McElhiney SP, Pachuk C, unpublished data]. In addition, the serum immunoglobulin isotype profile was different in mice immunized with constructs expressing secreted gD; expression of cell-associated gD was associated with a predominance of serum IgG2a, while higher serum levels of IgG1 were associated with expression of the secreted form of the antigens. While secretion of a variety of other antigens has also enhanced antibody responses [85], there are examples where no significant differences in immune response were seen in animals immunized with constructs expressing secreted versus cell-associated forms of antigen [86]. Perhaps the conflicting results can be explained by differences in serum level antigen concentrations resulting from differences in antigen expression or antigen stability.

As an alternative to secreting antigen from a cell via the use of secretory signals, the co-expression of proteins or peptides that induce cellular apoptosis may have merit. Not all proteins can be secreted from a cell, due to the presence of domains that interact with intracellular membranes. In these instances, induced apoptosis following antigen synthesis would allow spillage of antigen from the cell, resulting in increased antigen availability to APCs. In addition, cells undergoing apoptosis may recruit inflammatory cells to the site, and thereby increase antigen delivery to APCs. Consistent with this, a self-replicating RNA vaccine was recently demonstrated to be more efficacious than a traditional plasmid-based vaccine. The increased efficacy was postulated to be due to apoptosis of cells harboring self-replicating RNA.

It has recently been demonstrated that secreted antigen can be targeted directly to APCs by a further modification of the antigen. Boyle *et al* have made C-terminal antigen fusions to CTLA4 [87••]. CTLA4, a ligand present on activated T-cells, binds to B7 molecules expressed on the surface of APCs. The presence of CTLA4 on the antigen fusion protein should allow targeting of the antigen to B7-expressing cells, thereby increasing the efficiency with which antigen is taken up by APCs. Experimental data suggests that this was in fact achieved. Mice immunized with plasmids expressing the B7 targeted antigen had much higher antibody levels than those mice immunized with the non-targeted antigen: at 2 weeks post-immunization, mice receiving the CTLA4 fusion protein plasmid had antigen-specific antibody titers 10,000-fold higher than mice immunized with the native antigen plasmid. In addition, a 7000-fold increase in IgG1 levels was seen in mice immunized with the CTLA4 fusion construct as compared to mice immunized with the plasmid expressing the non-targeted antigen.

In addition to antigen modifications that allow targeting of antigen to specific cell types, modifications have also been incorporated which allow intracellular targeting of antigen. Expression of antigen as a fusion protein to lysosome-associated membrane protein (LAMP-1) has been shown to result in endosomal/lysosomal localization of antigen. LAMP-1 contains a localization signal that re-routes antigen into the lysosomal compartment and therefore into the MHC class II processing pathway. Immunization with constructs expressing LAMP-1-antigen fusion proteins has been shown to result in enhanced CD4+ presentation of antigen. In addition, immunization of mice with a vector expressing a LAMP-1/tumor antigen fusion protein was found to protect mice from tumor challenge, whereas mice vaccinated with a vector expressing the unmodified tumor antigen were not protected [88]. However, the use of LAMP-1 fusion proteins does not always result in increased immune response and more research must be done in this area before the utility of this approach can be assessed [89].

Other modifications can be made at the level of promoter choice. The use of dendritic cell- and macrophage-specific promoters would allow for the production of antigen in these APCs following uptake of plasmid DNA into these cells. It will be interesting to see if expression of antigen within APCs results in enhanced immune responses.

Molecular adjuvants and modulators of immune response

Of the different ways to modulate the immune response to DNA immunization, one of the most promising may be through the co-administration of 'biological' adjuvants such

as cytokines. Cytokines are molecules secreted mainly by bone marrow-derived cells that act in an autocrine or paracrine manner to induce a specific response in cells expressing the appropriate cytokine receptor. Other adjuvants include the costimulatory molecules that help signal T-cell activation and expansion. The large number of studies identifies this area of DNA vaccine research as one of the most reproducible and important areas for further development.

Codelivery of IL-2, IFNγ or IL-4 expression cassettes in BALB/c mice

Of the different ways to modulate the immune response to DNA immunization, one of the most promising may be through the co-administration of 'biological' adjuvants such as cytokines. Multiple laboratories have reported that co-injection of plasmids encoding cytokines can have a substantial effect on the immune response to plasmid-encoded antigen, for example, in multiple viral and cancer antigen systems [90-93].

Two important cytokines that have been examined for their immune modulatory activities are granulocyte-macrophage colony-stimulating factor (GM-CSF) a putative Th0 cytokine, as well as the prototypic Th1-inducing cytokine IL-12. IL-12 plays a critical role in the Th1 immune response mainly by inducing production of the Th1-associated cytokine IFNγ. In contrast, GM-CSF is a hematopoietic growth factor, which stimulates neutrophil, monocyte/macrophage and eosinophil colony formation. It also induces proliferation and differentiation of erythroid and megakaryocyte progenitor cells. GM-CSF also increases the antibody-dependent cell-mediated cytotoxicity of neutrophils, eosinophils and macrophages but has not been reported to have a direct role in the generation of CTL response *in vivo*.

The effect of co-expressing the genes for murine *IL-12* and *GM-CSF* with DNA vaccine cassettes for HIV-1 has been studied [94,95]. CTL responses were increased up to 5-fold using the IL-12 plasmids but little effect was observed on CTL induction by GM-CSF using equivalent doses of plasmids. Lower doses were frequently less impressive for cellular immune modulation. Humoral responses were also studied. In repeated experiments IL-12 suppressed specific antibody responses by 10 to 20%, while GM-CSF appeared to have the opposite effect, increasing antibody responses 4- to 6-fold. The boost in serological responses with GM-CSF correlated with increased neutralizing antibody responses. This humoral effect is likely the consequence of increased CD4+ Th activity specific for B-cell responses.

Activation and proliferation of Th-cells play a critical role in inducing both a humoral immune response via expansion of antigen-activated B-cells and a cellular immune response via expansion of CD8+ cytotoxic T-lymphocytes. In further adjuvant experiments, a dramatic increase in proliferation (4-fold) was observed in animals co-immunized with antigen plasmids and IL-12 plasmids. Interestingly, a similar and sometimes even more dramatic increase in the stimulation index was observed in animals co-immunized with antigen plasmid and GM-CSF plasmid. It was interesting that both treatments appeared to increase effective helper T-cell responses yet they clearly had different effects on the induction and maintenance of an antigen-specific CTL response. Recently, similar studies have been performed in a large primate chimpanzee model. In these studies increased proliferation was also observed with *GM-CSF* as well as *IL-12* gene co-immunization.

The activity of GM-CSF and IL-12 was compared with other Th1-type cytokines for their ability to enhance *in vivo* immunity. In these studies, GM-CSF, IL-12 and IL-2 all enhanced the T-cell proliferative response [96••,97••]. It was somewhat surprising that IL-2 and IL-15 had significantly different activities in this model. However, it suggests that a DNA vaccination approach can give important insight into the subtle differences associated with these similar but non-overlapping vaccine adjuvants *in vivo*. This became more evident as CTL responses were evaluated. In general, in several studies IL-12 was the best driver of MHC-restricted CD8+ CTL activity, however, IL-15 also demonstrated significant potency. Interestingly, IL-18, which can drive IFNγ induction in a similar manner to IL-12, was a poor adjuvant for CTL responses.

Studies have been performed to determine the effects of such adjuvants on survival in an animal challenge system. These studies used a herpes simplex virus (HSV) model system. The HSV system has the advantages of lethal mucosal challenge as well as titrateable challenge doses. The model grants insight into the general value of this approach. Surprisingly, all of the Th1 cytokines increased vaccine potency. These included IL-12, IL-2, IL-15 and IL-18. In comparison with Th2 cytokines the immune responses induced were entirely different and the survival profile was similarly different. Th2 cytokines delivered as vaccine adjuvants actually decreased survival supporting a direct effect on the memory response. In these studies there were discernable differences between the different test groups. IL-12 was always the best mediator of protection and survival from lethal challenge. Additionally, IL-12 as well as the aforementioned GM-CSF controlled pathogenesis as evidenced by the almost complete lack of herpes-induced lesions in surviving mice.

In conclusion, evaluation of the adjuvant effects of cytokine co-immunization suggests that while IL-12 may be the best candidate to date for enhancing the cellular immune responses to plasmid antigens encoded by DNA vaccine cassettes, IL-15 (through expanding CTL responses) and GM-CSF (through expanding T-helper responses) also significantly enhanced antigen-specific cellular immune responses. These results indicate that further combination studies examining the cytokine approach are warranted.

Codelivery of B7-1 or B7-2 expression cassettes in BALB/c mice

Although antigen is expressed at significant levels in muscle following im inoculation of plasmid DNA, it is well established that muscle cells do not express the costimulatory molecules required for efficient antigen presentation. Among the different costimulatory molecules, B7-1 (CD80) and B7-2 (CD86) have been investigated by a number of groups as possible vaccine adjuvants. The B7-1 and B7-2 molecules interact with the CD28/CTLA4 molecules on T-cells providing an important second signal

in addition to ligation of the T-cell receptor through the MHC peptide complex [98]. These two signals then facilitate expression of IL-2 receptor and progression of the T-cell through the cycle of immune activation. B7-1 and B7-2 are upregulated during the antigen presentation process, most likely following CD40/CD40 ligand interactions between T-cells and APCs.

The genes for *B7-1* and *B7-2* have been tested as vaccine adjuvants in a similar manner to the cytokine studies outlined above. In these studies dramatically greater levels of proliferation were observed in the mice splenocytes collected from animals immunized with antigen plasmids and pCD86 (a 6-fold increase). In contrast, CD80 exhibited more modest effects on T-cell proliferation than CD86. Examination of CTL responses revealed that the animals immunized with antigen plasmids alone or antigen pulse plus pCD80 showed low levels of CTL responses. In contrast, co-immunization with pCD86 resulted in a dramatic increase in CTL activity that also resulted in an increase in CTL precursor frequency.

These results are consistent with the hypothesis that codelivery of a costimulatory molecule with DNA dramatically improves immune responses by providing more efficient antigen presentation. It has recently been reported that APCs that take up DNA increase their expression of costimulatory molecules [52].

Codelivery of CD40 or CD40L expression cassettes in BALB/c mice

Costimulatory pathways are important avenues for gleaning new and functional DNA vaccine adjuvants. As B7-2 adjuvant activity likely involves subsequent induction of the CD40L, the effects of this pathway on immune induction have been investigated. The ability of CD40L, as well as CD40, to enhance the humoral and cellular immune responses to plasmid antigen vaccines has also been researched [Sin JI, Weiner DB, manuscript in preparation]. Co-immunizing mice with either B7-2 (CD86) or CD40L plus antigen expression cassettes led to enhanced antigen-specific T-cell proliferation and CTL activity. B7-1 and CD40 enhanced cellular immune responses to a much lesser extent. These results identify specific costimulatory pathways, particularly B7-2 and CD40L as important vaccine adjuvants for further studies. It is not unlikely that changing the specific antigen could have dramatic effects on vaccine-induced immune responses and should be considered in further investigation of this approach.

Conclusions

In conclusion, the potential of plasmid DNA-based vaccines has sparked enormous activity in DNA delivery research. It is recognized that one of the major limitations to the success of DNA vaccines is its delivery. Condensed and entrapped DNA designed for active transfection methods are predicted to yield consistent and desired levels of transfection. Advances in controlled self assembly of DNA complexing agents, novel carriers for oral, skin and lung administration are expected in the near future. Intradermal inoculations have shown promise in several animal species for certain antigens. However, the rules that govern why certain antigens and certain sites perform differently from others is unclear. Further research is expected to clarify these issues as well as to develop a clear understanding of the cell types involved. Unraveling the intracellular pathways that need to be invoked to assure robust immune responses, and developments in engineering of the immune response through chemokines, costimulatory molecules and antigen delivery should also allow rational design of delivery vehicles for plasmid DNA vaccines.

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Recent developments in mucosal delivery of pDNA vaccines

Andrew GC Barnes*, Christina Bamfield*, Robert Brew & Linda S Klavinskis

Address
Department of Immunobiology
Guy's, King's and St Thomas' Medical School (GKT)
St Thomas Street
London
SE1 9RT
UK
Email: linda.klavinskis@kcl.ac.uk

*Andrew GC Barnes and Christina Bamfield contributed equally to the review

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The development of DNA vaccination to mucosal surfaces has continued apace over the last 2 years, with the investigation of several novel delivery vehicles. There have been advances in the understanding of the basic immunological mechanisms behind the induction of immune responses by plasmid DNA. The mechanistic insights are paving the way for the design of a second generation of mucosally delivered DNA vaccines. This article reviews the recent progress in the field of microparticle, cationic lipid and bacterial delivery systems. All these mechanisms afford some protection from environmental degradation and facilitate DNA uptake. These methods have been compared with respect to transfection efficiency, ability to elicit a full range of immune responses and their relative safety for in vivo applications.

Keywords Cationic lipid, delivery, DNA, microparticle, mucosal, vaccine

Introduction

Mucosal immunization

Infectious diseases are associated with global morbidity and mortality. The majority of these diseases are caused by pathogens that first have to either cross or infect/colonize the mucosa prior to infection of the host. It is therefore logical to assume that pre-established pathogen-specific immunity at the site of entry could help prevent the establishment of clinical disease. Despite this, the Salk polio vaccine is the only commonly administered vaccine that is targeted to specifically generate mucosal, as well as systemic immunity [1].

Optimal induction of specific immune responses at the mucosa have in general been associated with targeting antigens to the specialized sites of the mucosal immune system. Such sites may include the organized lymphoid tissue associated with the gut, rectum, bronchus and nasopharynx or the draining lymph nodes [2]. Antigens presented at these sites induce the priming and homing of IgA-committed B-cells and effector T-cells to a variety of mucosal tissues. Such an approach has the advantage of potentially achieving both systemic immunity and secretion of antigen-specific IgA at distal mucosal sites. In contrast, with few exceptions, systemic vaccination strategies have failed to elicit significant mucosal immunity [3]. However, systemic vaccinations combined with mucosal administration regimes have been reported to boost mucosal immune responses [4,5•]. The large surface of the mucosal tissues is protected primarily by the secretory form of antibodies belonging to the IgA isotype [6]. Secretory IgA (sIgA) has been demonstrated to have neutralizing functions and the potential to block bacterial and viral adherence to epithelia [7]. In addition to sIgA, cellular proliferative and cytotoxic T-lymphocyte (CTL) responses may be essential for virus clearance and prevention of dissemination from the mucosal surface [8,9].

Induction of both cellular and sIgA responses can be achieved by direct delivery of antigens to the mucosal surface. However, mucosal surfaces are highly immunotolerent environments, mainly due to the high antigenic load commonly found at these sites, such as airborne particulates and food-derived peptides [3]. To overcome tolerance, adjuvants, such as cholera toxin sub-unit B (CTB), are often required to elicit potent immune responses to recombinant antigens [10-13]. A number of studies have demonstrated how mucosal immunization with recombinant antigens can induce protection against subsequent viral challenge [14,15].

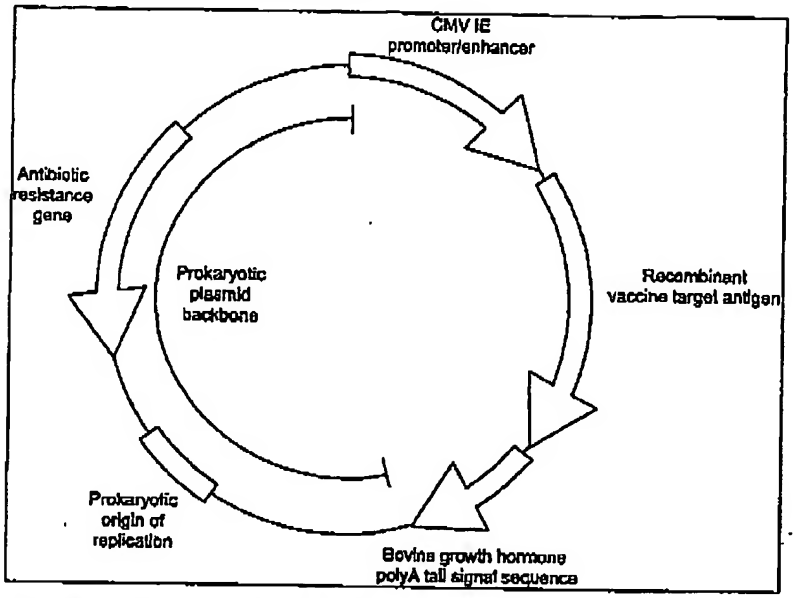
There is currently a need for an alternative to both recombinant protein and attenuated virus vaccines. There have been various risks associated with live attenuated virus vaccines. The secretion of virus has been reported after the administration of live polio vaccine or a combined regime of live and inactivated vaccine [16]. Alternatively, recombinant protein vaccines, often being derived from transformed bacteria, commonly show different protein folding patterns to the native protein. This can lead to the generation of antibodies with only a low affinity to the live pathogen. Furthermore, because of the exogenous nature of the vaccine antigen, immune responses tend to be heavily skewed in favor of T-helper 2 (Th2) responses [3]. The CTL response may also be weaker than following endogenous expression of the antigen from virus infection.

DNA vaccines

A recent development in vaccine technology is that of DNA vaccines, in which antigens are sythesized *in vivo* after direct introduction of their coding sequences into cells. DNA vaccines consist of a bacterial plasmid with a strong eukaryotic viral promoter, the gene of interest and a polyadenylation termination sequence (Figure 1). The immediate early (IE) promoter/enhancer of human cytomegalovirus (CMV) is one of the most frequently used. The IE promoter has been shown to drive high levels of expression of various reporter proteins, in a wide variety of mammalian cell types [17]. DNA vaccines represent a molecularly defined entity, which is both non-replicative *in vivo* (hence safe) and easily produced in large quantities. The safety of DNA vaccines has been studied from the earliest investigations. No *in vivo* evidence for chromosomal integration has been reported using PCR analysis of gel-purified genomic DNA extracted from different tissues [18,19].

Vaccination strategies using plasmid DNA (pDNA)-encoded proteins are particularly pertinent to the generation of immune responses to intracellular pathogens. The DNA-encoded protein

Figure 1. Schematic diagram of a typical DNA vaccine plasmid.



The schematic shows the different sequencing elements commonly found on a typical DNA vaccine plasmid. The cytomegalovirus immediate early (CMV IE) promoter/enhancer sequence initiates RNA polymerase II upstream of the 5' end of the target antigen sequence. The CMV IE sequence will promote transcription in mammalian cells but not prokaryotic. Downstream of the CMV IE sequence is the target antigen sequence orientated 5' to 3', which is flanked by the bovine growth hormone poly adenylation (BGH polyA) signal sequence. The BGH polyA sequence is a highly promiscuous polyA signaling sequence which promotes the synthesis of a 3' polyA tail (important for mRNA stability in mammalian cells). The origin of replication (usually ori E) allows the plasmid to maintain copy number in the prokaryotic transformant (usually *E coli*) used for cloning. Finally, there is a prokaryotic gene which confers antibiotic resistance to the bacterial transformant used for cloning. Many resistance markers can be used, the most common being the *bla* gene which codes for ampicillin resistance in *E coli*. Both the plasmid origin of replication and the antibiotic resistance encoding sequences form part of the prokaryotic plasmid backbone, rich in unmethylated CpG residues.

is expressed in the host cell and therefore undergoes appropriate post-translational modification and intracellular transport. The protein will also be presented by MHC class I and so can generate a CTL response, as well as high affinity humoral responses [20•]. Furthermore, unlike eukaryotic DNA, bacterial DNA contains a high frequency of unmethylated CpG dinucleotide sequences that elicit both innate and adaptive immune responses in vertebrates. These sequences induce dendritic and B-cell activation, upregulation of MHC class II, immunoglobulin and cytokine secretion [21,22,23••]. This means that prokaryotic-derived plasmids containing bacterial DNA backbones, may be natural adjuvants that can increase immune responses to the *in situ* expression of the encoded protein.

Initial studies demonstrated that direct intramuscular (im) injection of pDNA in saline (so called 'naked DNA') using reporter genes such as *luciferase* and *chloramphenicol acetyl transferase* led to long-term expression of the reporter gene [18,24]. Subsequent studies using pDNA encoding viral and bacterial antigens have demonstrated that it is possible to induce immune responses to the encoded protein. [25,26•,27]. Several studies have shown that direct im injection of naked pDNA encoding influenza or HIV proteins protected both mice and non-human primates respectively, after subsequent challenge with homologous or heterologous strains of virus [28,29].

A major drawback with using naked pDNA is that multiple doses of large amounts of DNA (1 to 2 mg) are required to induce optimal CTL and humoral responses in non-human primates [30,31••]. This is further exacerbated by the fact that compared to systemic routes of delivery, such as im inoculation; mucosal administration of naked DNA results in a greatly decreased uptake and longevity [31••]. This is probably due to endonucleases present in and the general dilution effects of mucosal secretions, which may decrease the effective concentration of pDNA at the mucosal surface [32, Klavinskis LS, unpublished observations]. However, recently a number of advances have been made, particularly with respect to pDNA delivery, that have greatly increased the efficacy of mucosal DNA vaccination. The four recent delivery systems that have been applied to mucosal DNA vaccine delivery include: ballistic delivery, cationic lipid complexes, microparticles and bacterial vectors.

Delivery of plasmid DNA to mucosal surfaces

Ballistic/jet delivery

The first significant improvement on manual injection of naked DNA was a technique termed 'gene gun'. The gene gun has been particularly effective at delivering pDNA intradermally. Gold microparticles are coated with pDNA in the presence of spermidine and are delivered to the skin by high velocity bombardment. This results in the

expression of the encoded protein in approximately 10 to 20% of dermal cells at the site of bombardment [33]. The gene gun delivers the pDNA directly into the cytosol and is associated with the induction of both humoral and cellular immune responses at lower concentrations of pDNA than by im injection [34•]. Gene gun inoculation of BALB/c mice with as little as 0.4 μg plasmid encoding influenza hemagglutinin glycoproteins could confer 95% protection against lethal infection with live virus, after just one boost [35]. Dermal gene gun inoculation with vaccine plasmid has been shown to be highly effective at inducing high titer antibody responses to a range of bacterial and viral pathogens [36]. These include inducing long-term protection from lethal rabies challenge after just one boost [26•]. Despite the high efficacy of the gene gun, it is a financially expensive method of vaccination, and to adapt it for induction of mucosal immunity requires highly invasive procedures. Such procedures include direct bombardment of the vaginal mucosa or the surgical exposure of the Peyer's patch prior to bombardment [37,38].

A more practical application of ballistic delivery to mucosal surfaces is the use of a high pressure jet injection (derived from dentistry) for the delivery of pDNA in solution to the buccal mucosa [39••]. The jet technique elicits antigen-specific sIgA in lung lavage and specific serum IgG antibody, predominately of the IgG2a isotype. Potentially this method affords a simple, safe and patient compliant method of mucosal vaccination.

Biodegradable particulate delivery systems

In attempts to overcome enzymatic degradation and improve antigen expression of pDNA, a variety of microparticles have been developed. Unlike gold particles, polymer microparticles, synthesized from poly(lactide-co glycolide) (PLG), are fully biodegradable [40]. These microparticles are produced using a solvent evaporation process. During the synthesis of the microparticles, therapeutic drugs or pDNA present in the aqueous-phase become encapsulated during the formation of the emulsion [41-43]. The PLG microparticles are taken up by cells and release their contents slowly as they are biodegraded (at a rate determined by particle size). The entrapped pDNA is protected from the external environment by the polymer, allowing mucosal administration without fear of degradation. However, the rate of uptake of PLG microparticles across the mucosal epithelium is known to be low (< 0.01%), which may limit the efficiency of this approach.

This technology has been used to engineer an orally administered rotavirus microparticle vaccine for preclinical testing in BALB/c mice [44•]. The DNA expression vector used in this study encoded the VP6 protein encoded by the EW strain of rotavirus under the control of the CMV IE promoter/enhancer. The mice were inoculated orally with PLG microparticles containing either VP6-encoding pDNA or control pDNA. Each mouse received a single dose of particles containing approximately 50 μg of pDNA. Antibodies specific to VP6 were detected in serum from 4 weeks post-inoculation. Antibody titers peaked at 6 weeks post-infection and were still present by 12 weeks post-infection. Low levels of fecal sIgA specific to VP6 were detected only after 6 weeks post-inoculation, which is surprising. Significantly lower virus titers were shed in the vaccinated mice compared to the controls, following virus challenge. However, the duration of virus shed remained the same and it was not possible to evaluate whether the vaccine protected the mice from clinical disease. Important questions still need to be addressed to confirm the efficacy of this new approach to DNA vaccination. However, if protection from clinical disease could be achieved with just a single inoculation, this would represent a major advancement in DNA vaccination.

By modifying the solvent evaporation process, cationic PLG microparticles can be generated [45•]. Either cetyltrimethylammonium bromide (CTAB), dimethyl dioctadecylammonium bromide (DDA) or 1,2-dioleoyl-1,3-trimethylammoniumpropane (DOTAP) were dissolved in the aqueous-phase or the PLG polymer solution prior to particle synthesis. The resultant cationic microparticles could then bind pDNA via ionic interactions between the particle surfaces (which are positively charged) and the negatively charged phosphate backbone of the DNA. From the three types of cationic particle produced, the PLG/CTAB particles had the highest surface charge density and bound pDNA the most efficiently. All three cationic microparticle beads were loaded with pDNA encoding HIV-1 p55 gag, driven off the CMV IE promotor/enhancer. The pDNA-bound microparticles were injected via the im route and compared with an equal dose (1 μg) of naked pDNA encoding HIV-1 p55 [45•].

The naked DNA failed to induce a CTL response after a single inoculation. By comparison, both the PLG/CTAB-*p55* pDNA and the PLG/DDA-*p55* pDNA induced a potent CTL response, above those observed in p55-expressing vaccinia infected mice. All three types of surface-bound pDNA microparticles induced high serum antibody titer 1 month after the booster vaccination. p55 gag-specific antibody titers were 2 to 3 $\log_{10}$ higher than were induced by naked vaccine vector alone. It has been argued that pDNA has greater stability when bound to the surface of a bead rather than encapsulated. This is due to the high shear that occurs at the organic-/aqueous-phase interface during encapsulation [46]. The *in vitro* release rate of pDNA from the cationic microparticles was initially rapid (35% by day 1) but then slowed until by day 14, 75% of the pDNA bound to the beads had been relinquished. The dynamics of the release properties are potentially closer to an acute virus infection than the encapsulated pDNA delivery systems. However, whether the cationic PLG microparticles offer a more potent mucosal delivery system remains to be seen. Singh *et al* [45•] allude to enhanced mucosal potency in their paper on systemic delivery. The mucosal data is awaited for critical review.

Cationic lipid complexes

Cationic lipid technology has provided one of the best-characterized non-viral delivery systems. The rationale was the ability of these lipids to facilitate transfer of the heavily charged pDNA into the cytosol. The lipids used to complex the pDNA consist of a positively charged lipid, eg, DMRIE [47-50] or GAP-DLRIE [48,51,52], which bind to the negative charge of the phosphate pDNA backbone. A neutral phospholipid is generally included as a colipid for stability. The formation of the pDNA-lipid complexes is in many

respects analogous to the cationic microparticles described previously. Successful gene transfer requires the condensation of the plasmid, cellular-binding and uptake [53]. Many different lipid formulations have been analyzed which enhance these processes in specific cell types [51,54].

Lipid formulations have mostly been applied to the delivery of pDNA vaccines to the respiratory and gastrointestinal tract [31••,49,55,56]. It has now been shown that incorporation of pDNA into lipid complexes can result in enhancement of both humoral and cellular immune responses following mucosal delivery [31,48]. This enhancement of the immune response has in part been attributed to an increase in expression of the encoded protein in mucosal tissues [31••]. Intranasal delivery of a reporter gene (firefly *luciferase*) complexed to DMRIE/DOPE was demonstrated to increase protein expression by 30-fold in nasal tissue compared to administration of the cognate naked pDNA [31••]. Analysis of the immune responses induced to the encoded protein demonstrated that immunization with pDNA-lipid complexes induced a significant increase in specific mucosal IgA antibody when compared with administration of naked pDNA [31••]. Following intranasal delivery of the pDNA-lipid complexes, specific sIgA was detected in vaginal and rectal fluids [49]. This observation provides clear evidence for the potential of mucosal delivery of pDNA at one site to induce immune responses at distal sites via a non-invasive route of vaccination. This may relate to the concept of the common mucosal immune system [57].

As discussed above, the lipids selected for complexing with pDNA may influence the transfection efficiency of specific cell types, and may act synergistically with bacterial pDNA in providing an adjuvant effect [Barnfield C, unpublished observations]. A comparison of humoral responses elicited by either intranasal or oral vaccination with DMRIE/DOPE or De Cholesterol/DOPE indicated that both antigen-specific serum IgG and antigen-specific IgA were significantly enhanced using DMRIE/DOPE [Barnfield C, Klavinskis L, unpublished observations]. This observation may relate in part to the level of protein expression achieved and the adjuventicity of the lipids [Barnfield C, Klavinskis L, unpublished observations]. These mechanistic insights into the mode of action of pDNA lipid complexes following mucosal administration, may explain why in certain studies using lipid-formulated pDNA-specific mucosal antibodies were only minimally detected [50,55].

Bacterial vectors

An alternative to lipid complexes or microparticles is the use of plasmid transformed live bacteria to deliver the pDNA to the mucosa. Presently four different bacterial species are being evaluated for pDNA delivery; *Shigella flexneri*, *Salmonella typhi*, *Listeria monocytogenes* and invasive strains of *Escherichia coli* [58]. Integration of the pDNA into the genomes of cell lines following *in vitro* infection with pDNA transformed *E coli* and *L monocytogenes* have been reported. However, it is not known whether integration occurs *in vivo*. Clearly safety concerns will have to be fully addressed prior to clinical trials using bacterial pDNA delivery vectors in man.

A highly attenuated strain of *Shigella flexneri* has been developed and applied to deliver a measles virus (MV) DNA vaccine in a murine study [59••]. The *Shigella flexneri* strain used contained a deletion (Δ) in the *asd* gene. This gene encodes an aspartate β-semialdehyde dehydrogenase, which is essential for cell wall synthesis and bacterial growth. Between 10^7 and 10^8 colony forming units (cfu) could be safely inoculated (in) into the lungs of both normal and immunocompromised (γ-interferon knockout and severely combined immunodeficient) BALB/cJ mice. Despite being replication defective, the *Δasd* mutants retain the ability to escape the lysozome and deliver the pDNA directly into the cytosol, following cellular uptake [60]. The bacteria were cleared from the lungs of all the mice within 3 days of inoculation, demonstrating the lack of reversion back to virulence by the *Δasd* mutants. Conversely, similar titers of wild-type *Shigella flexneri* inoculated into the same three strains of mice proved 100% lethal by 5 to 6 days post-inoculation.

For the purposes of vaccination the *Δasd Shigella flexneri* mutants were transformed with a DNA vaccine plasmid encoding either the *MV fusion*, *hemagglutinin* or *nucleoprotein* gene driven by the CMV IE promotor/enhancer. Test mice were inoculated intranasally, with 1 to 3 x 10^9 cfu and then boosted monthly. The immune responses induced to the MV-encoded proteins were predominantly but not exclusively Th1, since IL-4 was produced by splenocytes following *in vitro* restimulation with the appropriate MV protein. Both IgG and IgA responses to MV were detected in the serum of vaccinated mice, with titers increasing after the second boost. Antibody titers however, remained relatively low. Conversely, vaccination generated strong CTL responses after one boost. Surprisingly, the CTL responses were equivalent to the levels seen in mice inoculated by the systemic route with attenuated *Salmonella typhi* harboring the same MV-encoding plasmid. Furthermore, immune responses were only slightly reduced in mice vaccinated against *Δasd Shigella flexneri* prior to vaccination with MV strains. This study clearly highlights the potential of using bacterial DNA vaccine delivery systems, especially with respect to the third world.

Conclusion

The possibility of introducing mucosal immune responses to a protein expressed directly from an introduced gene by various modes of pDNA delivery represents an attractive alternative to attenuated or recombinant vaccines. Mucosal delivery of pDNA vaccines by intranasal, buccal or oral routes represent simple, non-invasive routes which are highly suited for mass vaccination. The immune responses induced after mucosal delivery of DNA vaccines have in general been lower than those induced after parental delivery. However, with improved mechanisms of delivery, increased antibody as well as cellular responses can now effectively be induced. A number of new delivery techniques are being employed which enhance antigen expression and subsequent immune responses, ie, microparticles, cationic lipid aggregates and bacterial carrier vectors. It is becoming apparent that the type of immune response elicited by mucosal application of pDNA is influenced by several factors including the mucosal site, delivery vehicle and incorporation of adjuvants. The second generation of DNA vaccines both protect pDNA from mucosal endonuclease degradation and enhance cellular uptake. With the exception of the Salk polio vaccine [1],

there is currently an absence of clinically administered mucosal vaccines. The increased efficacy of mucosal DNA vaccines may make these vaccines realistic candidates for clinical trials in the future.

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DNA vaccination strategies against infectious diseases

Allison M. Watts, Ronald C. Kennedy *

Department of Microbiology and Immunology, The University of Oklahoma Health Science Center, 800 North Research Parkway, Suite 462, Oklahoma City, OK 73104, USA

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Abstract

DNA immunisation represents a novel approach to vaccine and immunotherapeutic development. Injection of plasmid DNA encoding a foreign gene of interest can result in the subsequent expression of the foreign gene products and the induction of an immune response within a host. This is relevant to prophylactic and therapeutic vaccination strategies when the foreign gene represents a protective epitope from a pathogen. The recent demonstration by a number of laboratories that these immune responses evoke protective immunity against some infectious diseases and cancers provides support for the use of this approach. In this article, we attempt to present an informative and unbiased representation of the field of DNA immunisation. The focus is on studies that impart information on the development of vaccination strategies against a number of human and animal pathogens. Investigations that describe the mechanism(s) of protective immunity induced by DNA immunisation highlight the advantages and disadvantages of this approach to developing vaccines within a given system. A variety of systems in which DNA vaccination has resulted in the induction of protective immunity, as well as the correlates associated with these protective immune responses, will be described. Particular attention will focus on systems involving parasitic diseases. Finally, the potential of DNA immunisation is discussed as it relates to veterinary medicine and its role as a possible vaccine strategy against animal coccidioses.

Keywords: Animal coccidioses; DNA immunisation; DNA vaccines; Parasitic infections

1. Historical perspectives of nucleic acid immunisation

Concepts related to gene delivery for therapeutic modalities began with the DNA transfer experiments that were initiated and described in the 1950s. In these studies, crude DNA preparations isolated from neoplastic tumours were shown to induce in vivo tumour formation when inoculated into rodents. Experiments decades later demonstrated that the direct injection of DNA without any contaminating material resulted in the in vivo expression of DNA encoded gene products. The potential for DNA immunisation as a means of inducing an antigen-specific immune response had become apparent (reviewed in [1]).

The ability of DNA to induce an immune response in vivo may have first been demonstrated in 1962 [2]. In these investigations, the

* Corresponding author. Tel.: +1-405-271-5630; fax: +1-405-271-6339; e-mail: ronald-kennedy@ouhsc.edu

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s.c. inoculation of crude preparations of polyomavirus DNA containing transforming sequences into newborn hamsters resulted not only in the induction of tumours, but also in the production of antibodies specific to the virus. However, it was unclear whether the tumour induced by the DNA was responsible for the induction of the anti-viral antibodies or whether the DNA inoculation itself generated the immune response. Other investigators demonstrated that inoculation of polyomavirus DNA resulted in more hamsters developing anti-viral antibodies than tumours [3], suggesting that the DNA itself could induce an anti-viral specific immune response. These early observations were later confirmed by inoculation of mice and hamsters with recombinant forms of polyomavirus DNA [4]. Together, these studies suggested that immune responses may be induced as a result of DNA injection (reviewed in [1]).

In the early 1980s, two independent groups of investigators demonstrated that the expression of plasmids encoding hepatitis B viral proteins and insulin could induce an immune response when injected into animals [5,6]. However, the major turning point regarding the in vivo expression of a foreign gene inserted into a plasmid via direct DNA inoculation was reported in 1990 by Wolff et al. [7]. Utilising chloramphenicol acetyl transferase, luciferase, or β-galactosidase as the foreign reporter gene, these investigators demonstrated that inoculation of either purified RNA or DNA into the skeletal muscle resulted in expression of the enzyme. The detection of episomal DNA by Southern blot analysis 30 days p.i. and the presence of enzyme activity 60 days after injection provided evidence that the foreign reporter genes were being taken up and expressed in vivo. That the majority of the injected DNA persisted as extrachromosomal episomal DNA within the muscle may have potentially been the result of the low proliferative state of the myocytes and/or reflected the structural features of the muscle that make it particularly suited to uptake polynucleotides. However, the authors were not able to rule out the possibility that low levels of chromosomal integration of the injected DNA had occurred. It was not known whether these transcribed and translated gene products could be presented to the immune system and generate an immune response.

In 1992, Tang et al. [8] found that plasmids coated onto gold beads resulted in foreign gene expression and the induction of an antibody response to the foreign gene product in mice. They used genetic immunisation as a means of generating a humoral immune response to a gene product by injecting plasmid DNA encoding the foreign gene into a host. Following this report, a number of other investigators employed plasmid DNA immunisation to induce humoral and cell mediated immune responses to influenza A virus [9,10], human immunodeficiency virus (HIV) [11], and hepatitis B virus surface antigen [12], and collectively demonstrated the potential for DNA immunisation as a vaccine strategy against viral pathogens. The potential of DNA immunisation as a method for cancer immunotherapy was also reported [13–16]. Other early investigations also demonstrated the use of DNA immunisation for the induction of antibodies to immunoglobulin light chains [17]. Thus, it was clear that immunisation with DNA could generate antigen-specific immune responses to a specified gene product, and could therefore potentially serve as an immunotherapeutic modality.

2. Delivery of DNA vaccine

From a simplistic view, DNA vaccination requires that the plasmid DNA enters a cell, be transcribed and translated, and the foreign gene product be presented as an antigen in tissues accessible to the immune system. For successful transfection of the DNA and expression of the antigen, the plasmid should include an efficient promoter to drive transcription of the encoded antigen. A number of promoters are suitable to drive transcription. These can include sequences derived from genomic DNA which provide efficient autonomous replication in a variety of mammalian cells. Many plasmid DNA immunisation schemes to date employ strong viral promoters, one of the most efficient being the

human cytomegalovirus (CMV) immediate/early promoter. The optimal plasmid should be in a supercoiled state and contain a 3′ poly A tail to ensure the stability of the transcribed mRNA. Most plasmids also encode a poly A sequence such as the SV40 late poly-adenylation signal.

The site of inoculation plays an important role in the induction of protective immune responses to DNA vaccination. The most easily accessible tissues for DNA immunisation are the exterior skin and mucosal surfaces of a host. In studies evaluating route of delivery, DNA inoculation induced protective immune responses when administered mucosally to the nasal passages or trachea of experimental animals. A significantly higher level of protection was obtained when the DNA was delivered to the skin by methods that enhanced uptake of the DNA and increased the transfection efficiency [10]. Although cardiac muscle, liver, and dermis have been shown to express gene products after DNA inoculation, the optimal response is seen with inoculation into skeletal muscle. In direct comparative studies in mice, i.m., i.v., intranasal, intradermal, and s.c. routes of inoculation of DNA induced some protection against a lethal influenza virus experimental challenge [10], but the i.p. route did not. The i.m. and i.v. routes of inoculation resulted in the best responses within this system. In chickens, the i.m., i.v., and mucosal administration of DNA provided some protective immunity against a lethal challenge with avian influenza virus [10], with little to no protection being observed by s.c., i.p., intrabursal, and intraorbital routes of inoculation. Thus the route of DNA inoculation is important in the induction of protective immune responses, and the optimal route may vary with the system being examined.

The in vivo transfection efficiency of DNA has been enhanced by, for example, adsorption of DNA to gold particles and delivery through the use of a gene gun [8,10] that bombards the skin with gold particles containing the plasmid DNA. The gold particles directly penetrate the skin due to the force of delivery, thereby increasing the rate of transfection without having to rely on the uptake of DNA by the host cell itself. At least 100-fold less plasmid DNA is required for the induction of protective immune responses when administered adsorbed to gold particles and delivered by a gene gun when compared with inoculation of plasmid DNA in saline using a syringe and needle; as little as 0.4 μg of DNA can induce protective immunity in mice when compared with hundreds of micrograms of DNA in saline administered without a gene gun [10]. Additional techniques utilised to enhance the in vivo uptake of inoculated DNA include the use of local anaesthetics, such as bupivacaine (1-butyl-*N*-(2,6-dimethylphenyl)-2-piperidinecarboxamide), myotoxins such as cardiotoxins, and a variety of ionic and lipid-based delivery systems (reviewed in [1]). However, in a number of systems the use of saline alone as a carrier moiety for DNA immunisation has resulted in the induction of specific immune responses and appears to be a reasonable approach if large quantities of plasmid DNA are available (reviewed in [1]). It is anticipated that studies on the most effective way to deliver plasmid DNA to the immune system for the induction of an immune response will continue to evolve and new delivery systems and vehicles will be evaluated.

3. Presumed mechanisms for DNA immunisation and immune responses

Cell-mediated immune responses can be divided into major histocompatibility complex (MHC) class I- or class II-specific. The MHC class I pathway presents endogenously synthesised protein antigens that are initially translated on the ribosomes of the endoplasmic reticulum (ER) or cytosol. The proteins are then processed into peptides and associated with MHC class I molecules in the lumen of the ER. A variety of other accessory molecules, including the transporter proteins TAP-1 and TAP-2, assist in the targeting and association of the peptide antigen with the MHC class I molecules (Fig. 1). The peptide–MHC class I complex is transported to the cell surface where it is recognised by the T-cell receptor (TCR) expressed on $CD8^+$ T cells. Activated $CD8^+$ cytotoxic T lymphocytes (CTL) then target MHC class I antigen present-

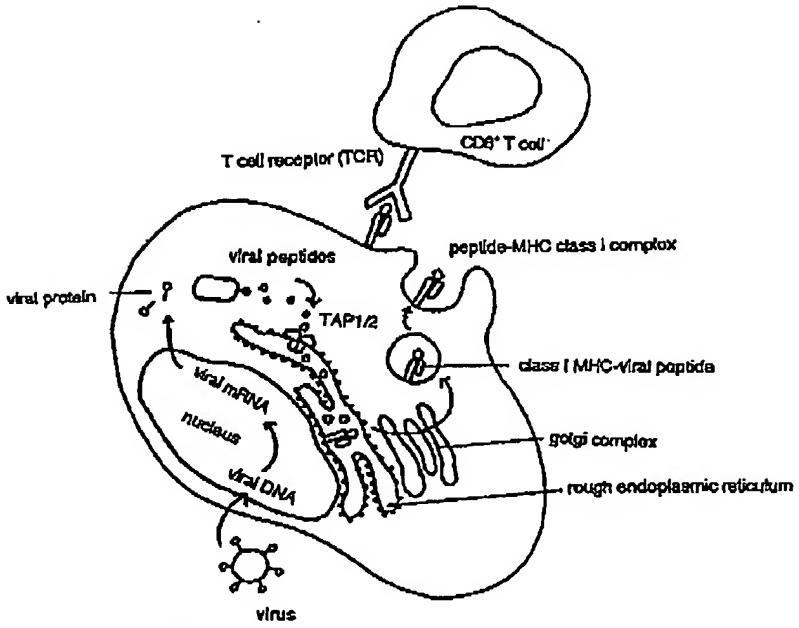

Fig. 1. In the MHC class I pathway of antigen presentation, endogenously synthesised proteins (such as products of viral transcription/translation) are processed into peptides in the cytoplasm and are then actively transported into the lumen of the rough endoplasmic reticulum by TAP 1/2 transport proteins. Within the lumen, peptide associates with the MHC class I molecule. The complex is transported out through the Golgi complex to the cell surface where it is then recognised by the $CD8^+$ T-cell receptor. Recognition leads to activation of a cell-mediated CTL immune response specific for the antigen presented.

ing cells and subsequently lyse them. Also associated with MHC class I-restricted responses are $CD8^+$ T cells with suppressor activity. When activated, these cells can induce a state of unresponsiveness and/or anergy. With regard to vaccination, the induction of CTL responses is important when the organism in question is an intracellular pathogen. Activation of the endogenous pathway and the induction of MHC class I-restricted responses can result from infection of a cell by a pathogen, an attenuated vaccine that replicates in host cells, or a replicating vector that infects the cell prior to synthesis of the inserted foreign protein.

In contrast to the MHC class I pathway, the MHC class II pathway presents antigens that are processed exogenously. Extracellular proteins are acquired by endocytosis and are processed in endosomes into antigenic peptides that associate with MHC class II molecules. The peptide-MHC class II complex is transported to the cell surface where it is recognised by the T-cell receptor (TCR) expressed on $CD4^+$ T cells. The $CD4^+$ T cell becomes activated with the aid of a number of co-stimulatory molecules on the surfaces of both $CD4^+$ T cells and MHC class II antigen presenting cells. A $CD4^+$ T cell may then act as a T-helper lymphocyte and activate antigen-specific B cells to secrete antibody (Fig. 2). Inactivated and protein subunit component vaccines are predominantly presented by MHC class II pathways. The induction of MHC class II pathways by a particular vaccination strategy is appropriate when the pathogen is extracellular. Such is the case with cell-free virus or bacteria where neutralising or opsonising antibodies mediate protective immunity.

The $CD4^+$ T-cell population has been subdivided into T_H1 and T_H2 subsets based on the profile of cytokines secreted following activation.

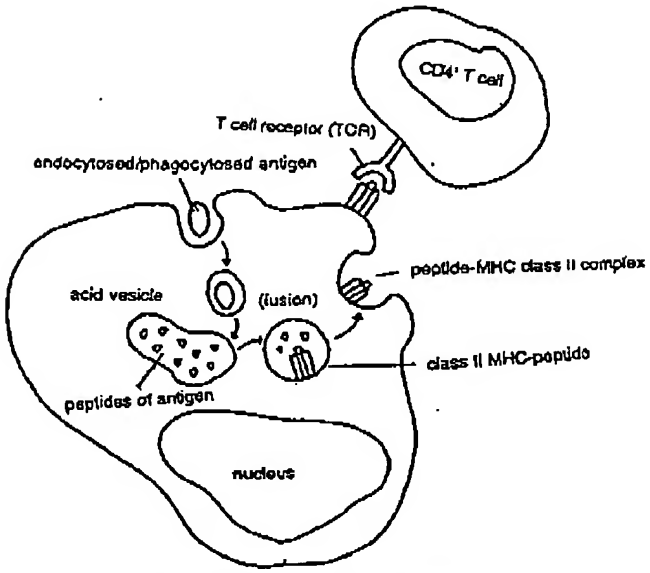

Fig. 2. In the MHC class II pathway of antigen presentation, endocytosed/phagocytosed protein antigens are processed into peptides within acidic endosomes. The endosomes then fuse with vesicles containing the MHC class II molecule, resulting in MHC class II–peptide complex formation. The complex is transported to the cell surface, where it is recognised by the $CD4^+$ T-cell receptor. Recognition leads to the activation of T helper lymphocytes which may produce an inflammatory reponse (T_H1), or antibody secretion by B cells (T_H2).

Among the cytokines secreted by the T_H1 $CD4^+$ cells are interferon-gamma (IFN-γ) and interleukin-2 (IL-2). Secretion of these cytokines results in T-cell proliferation and upregulation of MHC class II expression. The T_H1 $CD4^+$ cells seem to contribute only limited help for B cells in antibody secretion, yet they provide potent cell-mediated immune responses by stimulating CTL and increasing the phagocytic activity of monocytes and macrophages. Alternatively, the T_H2 $CD4^+$ T-cell population secretes cytokines such as IL-4, IL-5, IL-6, and IL-10 which cause the activation of B cells and induction of antibody isotype switch and secretion. These cells play a major role in supplying B cell help in the generation of a humoral immune response.

Other cytokines produced by T cells can modulate the immune response by directing it to either the T_H1 or T_H2 pathway; an outcome of direct manipulation of the immune response by co-injection of cytokine genes in association with DNA immunisation [18]. The co-delivery of IL-12 with a plasmid encoding HIV gp160 induced splenomegaly in mice, along with a reduction in antibody responses, and a dramatic increase in CTL activity. From this observation it was concluded that IL-12 suppresses T_H2 pathways and stimulates T_H1 responses. Co-delivery of granulocyte–macrophage colony stimulation factor (GM-CSF) resulted in an increase in antibody responses (activates T_H2) and did not appear to enhance CTL activity [19]. Therefore, it has become clear that the immune response generated by immunisation can be directed to a humoral or an inflammatory response with the co-administration of select cytokines.

4. Plasmid DNA immunisation and immunostimulatory properties

Studies based on antibody isotypes and cytokine secretion profiles have demonstrated that plasmid DNA immunisation primes T_H1

responses [20]. Mice injected with DNA encoding β-galactosidase showed IgG2a antibody isotype secretion and $CD4^+$ T-cell secretion of IFN-γ, but not IL-4 and IL-5. In contrast, mice given protein in saline or alum produced IgG1 and IgE β-galactosidase-specific antibodies and $CD4^+$ T-cell secretion of IL-4 and IL-5, but not IFN-γ. It was concluded that DNA immunisation induced a predominant T_H1 response, whereas protein immunisation induced a T_H2 response to the same antigen. Subsequently, short immunostimulatory sequences (ISS) were identified within the plasmid DNA sequence that enhanced the immunogenicity of the plasmid [21]. The ISS contain a CxG dinucleotide sequence in a particular base context, and this has also been referred to as the CpG immunostimulatory motif. Human monocytes transfected with plasmid DNA or double-stranded oligonucleotides containing the ISS transcribed large amounts of IFN-α, IFN-β, and IL-12 [21]. The ISS are not necessary for DNA immunisation to evoke antigen-specific immune responses, but they may alter or interfere with a particular immune response by inducing proinflammatory cytokines that can modulate immunologic pathways involved in protective immunity. However, it remains to be determined whether ISS exclusively target T_H1 pathways.

5. Studies involving DNA vaccines against human pathogens

Many DNA vaccines that target viral pathogens (reviewed in [1,22]) have been examined. Influenza virus was one of the first DNA immunisation experimental models to be evaluated. The specific antigens employed to target the immune response against influenza virus are the viral haemagglutinin (HA) and viral nucleoprotein (NP). Inoculation (i.m.) of mice with plasmid encoding the HA and NP genes induced HA inhibiting antibodies and CTL responses [23] and protection from experimental influenza virus challenge correlated with the amount of DNA injected and the titres of HA inhibiting antibody. Immunogenicity studies with nonhuman primates also revealed induction of HA inhibiting antibodies, though at lower levels than reported in mice [24].

Immunisation with plasmid DNA encoding HIV-1 gp160 induced humoral and cell-mediated immune responses in mice and subsequently, in cynomolgus monkeys (*Macaca fascicularis*) [25]. Both humoral and cell-mediated immune responses to HIV-1 gp160 were observed. The cynomolgus monkeys were challenged with a SHIV chimeric virus expressing the HIV-1 envelope gene encoding gp160, the simian immunodeficiency virus (SIV) core, and other structural and regulatory genes. Of the four DNA vaccinated and SHIV challenged monkeys, one developed a viral load similar to control animals, two developed transient viraemia and cleared the infection within 60 days, and one was completely protected. To evaluate the protective capacity of DNA vaccines against an experimental challenge with HIV-1, chimpanzees were immunised with a DNA vaccine preparation encoding the *env*, *rev*, and *gag/pol* genes from HIV-1 [26]. The immunised chimpanzees developed HIV-1 specific humoral and cell-mediated immune responses. Chimpanzees were challenged with a heterologous isolate of HIV-1 and, although both DNA vaccinated chimpanzees were protected from the establishment of infection, a transient viraemia was observed when compared with a single control infected animal. These studies implicate the protective potential of DNA vaccination strategies in a nonhuman primate model of experimental infection with HIV-1.

The potential of DNA vaccination as an immunotherapeutic modality in experimentally infected chimpanzees was also evaluated [27]. One HIV-1 infected chimpanzee was inoculated with plasmid DNA encoding the *env* structural and *rev* regulatory gene and a second was inoculated with a control plasmid DNA. The therapeutically HIV-1 DNA vaccinated chimpanzee demonstrated an enhanced humoral immune response and a decrease in viral load at week 20 following plasmid DNA injection, and remained at background levels during the course of the study. Although the therapeutic effectiveness could not be determined with such a small sample size, the authors concluded that the

immune response can have a direct impact on HIV-1 replication in chimpanzees.

Other investigators evaluated a DNA vaccination strategy within the SIV system. The SIV–macaque monkey model was utilised because it has many similarities with HIV-1 infection in humans and the sequelae to AIDS. Immunisation of macaques with plasmid DNA encoding either the SIV *env* gene receptor binding site or the extracellular domains of the *env* gene resulted in the induction of specific antibodies and $CD8^+$ MHC class I restricted CTL [28], although the animals were not protected from an experimental SIV challenge. Although these two sets of studies were contradictory with regard to the induction of protective immunity within the HIV-1/SIV primate model systems, it should be pointed out that the plasmid DNA constructs used in the HIV-1 and SIV studies were different. Additionally, the experimental challenge systems employed different monkeys and various HIV/SIV infectious inocula to evaluate protection from infection and/or disease. It remains to be determined whether DNA encoding HIV gene products will evoke protective immunity and prevent AIDS in humans.

Herpes viruses are a significant human pathogen and, despite decades of studies and years of evaluation, no commercial licensed vaccine is available. A repeatedly observed problem with the development of a vaccine against herpes simplex virus (HSV) in human clinical trials is that placebo vaccination may give up to 35% protection. Herpes simplex virus also represents a human pathogen where both prophylactic and post-exposure therapeutic vaccination strategies are appropriate. At least four groups of investigators have described the induction of HSV-specific immune responses and protection in animal models following injection with DNA encoding HSV proteins [29–32]. DNA immunisation with plasmids encoding a HSV-1 regulatory protein, designated ICP27 or the structural gene for the major glycoprotein gB, generated an immune response that resembled for the most part the immune response following exposure to replicating virus in mice [29,33]. Immunisation with gB encoding plasmid DNA induced a neutralising antibody response where the IgG2a isotype was dominant. The $CD4^+$ T-cell response had predominantly a T_H1 cytokine profile, but a CTL response was not detected, suggesting that a T_H1 cytokine profile is not always associated with a CTL-specific cell-mediated immune response. ICP27 DNA immunisation resulted in the induction of CTL response, but no specific antibodies were detected. To evaluate protective immunity, a murine model (zosteriform model) which results in the development of cutaneous lesions following HSV-1 challenge was employed. The cutaneous lesions are the result of viral dissemination along sensory nerves following replication in the nerve ganglion. Protection following either ICP27 DNA or gB DNA immunisation was observed in mice challenged with a low dose of HSV-1, but was not observed with a 50-fold higher challenge inoculum. In contrast, animals immunised with either an infectious virus or recombinant vaccinia virus expressing the HSV proteins were solidly immune to both the high and low dose HSV-1 challenge (reviewed in [34]). Protective immunity correlated with $CD4^+$ T cells as protein could be transferred adoptively in vivo. These data indicate that an attenuated vaccine or recombinant replicating vector strategy induce more complete immunity in an animal model system compared with DNA approaches.

The human microbial pathogen *Mycobacterium tuberculosis* is also a subject of DNA vaccination strategies. Even with antibacterial drug treatment and vaccination with an attenuated *Mycobacterium bovis* variant, bacille Calmette–Guerin (BCG), it has been estimated that there are still 10 million new cases of tuberculosis (TB) every year [35,36]. Tuberculosis occurs primarily in developing countries and results in 3 million deaths worldwide each year. With the development of multi-drug-resistant strains, and the ineffectiveness of the BCG vaccine in inducing protective immunity, DNA vaccination strategies are being actively examined. Immunisation with plasmid DNA encoding a secreted component of *M. tuberculosis*, designated antigen 85, generates humoral and cell-mediated immune responses in mice [35]. DNA vaccines that encode the *Mycobacterium leprae* heat shock protein, hsp65,

which is highly conserved among *Mycobacteria* species and appears antigenically similar to that of *M. tuberculosis*, has given similar results in mice [36]. Both DNA vaccines induced protective immunity in mice following experimental challenge with live *Mycobacterium*, and although protection induced by the hsp65 DNA vaccine was comparable with that arising from BCG immunisation, immunising with the protein alone failed to generate protective immunity against infectious challenge. Studies involving the use of DNA vaccines against a variety of microbial pathogens have highlighted the success and failures of this approach for inducing protective immunity in comparison with more conventional vaccine approaches (reviewed in [34]). It is apparent that in a number of situations DNA vaccination strategies require further refinement to improve immunogenicity and the induction of protective immunity.

6. Studies of DNA vaccines involving parasitic diseases

DNA immunisation against the malaria parasite has been extensively studied. The potential efficacy of a DNA vaccination strategy utilised the *Plasmodium yoelii* rodent malaria model [37], and mice were immunised i.m. with one of two different plasmids containing the *P. yoelii* circumsporozoite (CSP) gene. Both humoral and cell-mediated immune responses were induced after challenge with a high dose of sporozoites. A significant reduction in the number of liver stage schizonts was found in DNA vaccinated mice compared with mice given irradiated sporozoites. With a low challenge dose, sterile immunity was observed in some of the DNA immunised mice. The correlate of immunity was shown to be associated with $CD8^+$ T-cells as treatment of immunised and protected mice with a monoclonal anti-CD8 completely abolished protective immunity and $CD8^+$ CTL were probably responsible for eliminating *Plasmodium* infected hepatocytes. Encouragingly, inoculation of DNA containing genes from different parasite stages can bypass the genetic restriction of the immune response in mice that was observed with protein immunisation [38]. $CD8^+$ T cells, IFN-γ, and nitric oxide were also found to play a role in the protective immunity against malaria in mice. More recently, a mixture of four DNA plasmids encoding multiple antigens from *Plasmodium falciparum* was used to immunise rhesus monkeys (*Macaca mulatta*) [39]. The majority of immunised monkeys given either the individual plasmids or a combination of plasmids generated antigen-specific CTL, providing a foundation for the evaluation of multigene based immunisations to protect against malaria in humans. Immunisation with DNA plasmid encoding the CSP gene from *Plasmodium berghei* and expressing different amounts of the CSP were given by various routes of inoculation [40]. The strongest humoral immune response and the greatest level of protection was induced by epidermal vaccination with the high level CSP expressing plasmid. The immunisation protocol also utilised a gene gun to deliver three immunisations at 6 week intervals. The predominant antibody response to CSP was the IgG2a subclass suggestive of a T_H1 response and the authors concluded that the interval dependent induction of these antibodies by epidermal immunisation contradicted the concept that antibody responses induced by this method result in a T_H2-dependent response.

The enhanced immunogenicity for $CD8^+$ T-cell induction and protective immunity in mice primed with DNA immunisation (encoded pre-erythrocytic antigens from *P. berghei*) and boosted with a vaccinia virus vector (MVA) expressing the same malarial antigen has been described [41]. Thus DNA immunisation can be utilised with other vaccination modalities to enhance the immune response when compared with that generated by a single vaccination strategy alone. Such a prime-boost strategy was highly effective at inducing protective immunity associated with $CD8^+$ T cells in mice. It was of interest to note that when the immunisation was reversed, no protective immunity was observed.

The use of somatic transgene immunisation (STI) has also recently been described to provide immunity against *P. falciparum* malaria sporo-

zoites in mice [42]. Somatic transgene immunisation is an alternative approach to DNA based vaccination and is induced with transgenes under the control of lymphoid tissue specific regulatory elements. This approach requires direct inoculation of lymphoid tissues, such as the spleen, to allow long-term expression of the transgene in vivo. This represents a new modification of DNA immunisation technology that requires further evaluation to determine its effectiveness as an immunisation vehicle for vaccine delivery.

Schistosomes are trematode parasites of mammalian species that infect approximately 200 million people worldwide. The efficacy of nucleic acid immunisation for inducing protective immune responses against *Schistosoma japonicum*, the Asian blood fluke, has been evaluated in murine models. Mice were immunised with a variety of plasmids encoding a number of different gene products produced by schistosomes. These genes included the glutathione-*S*-transferase (GST), calreticulin, glyceraldehyde-3-phosphate dehydrogenase, an unidentified membrane associated antigen, a 14 kDa fatty-acid binding protein, fragments of paramyosin, full-length paramyosin, and a fusion between paramyosin and GST [43]. The paramyosin encoding DNA plasmids all induced antibodies to anti-paramyosin, and the paramyosin–GST gene fusion. None of the other DNA plasmids induced detectable antibody responses. The anti-paramyosin antibodies failed to protect mice from a challenge with *S. japonicum* cercariae.

Other investigations have described DNA immunisation to induce specific antibody responses to the 28 kDa GST protective antigen from *Schistosoma mansoni*. Three injections of 200 μg of plasmid DNA encoding the 28 kDa GST at 14 day intervals into the skin of rats resulted in production of IgG antibodies [44]. Sera from the immunised rats mediated antibody-dependent cell-mediated cytotoxicity in vitro with specific killing of parasite larvae. Challenge with *S. mansoni* infectious cercariae resulted in a boosting of the IgG response in DNA immunised animals, although protection against infection was not observed. Investigations have also shown that DNA immunisation of mice with plasmids expressing the 28 kDa GST from *S. mansoni* induced both humoral and cell-mediated immune responses against *S. mansoni* 28 kDa GST [45]. Plasmid DNA encoding a 20.8 kDa tegumental antigen expressed on *S. mansoni* sporocysts and adult worms has also been used to immunise mice [46]. Following immunisation with DNA encoding the 20.8 kDa tegumental antigen and subsequent challenge with infectious cercariae, a 30% reduction in worm burden was observed compared with control immunised and challenged mice. Individual plasmid DNA encoding the *S. mansoni* glutathione peroxidase (GPX), the cytosolic superoxide dismutase (SOD) (C-SOD), and the signal peptide containing SOD (SP-SOD) genes have been used to immunise groups of mice [47]. Following DNA immunisation and infectious challenge, worm burden reductions were 55%, 61%, and 45% for the GPX, C-SOD, and SP-SOD DNA immunised groups of mice, respectively, when compared with plasmid DNA immunised controls. This study suggests that plasmid DNA vaccination may afford some immunity against an experimental *schistosome* challenge in an animal model system.

Leishmaniasis is a parasitic disease that occurs in most parts of the world. An incidence of approximately 400 000 new cases per year has been reported and the worldwide prevalence of the disease is thought to be about 12 million cases. Leishmaniasis is caused by several species of intracellular protozoan parasites found in the genus *Leishmania*. There are three main categories of leishmaniasis: cutaneous, mucocutaneous, and visceral leishmaniasis. One of the early successes of genetic immunisation was protection of mice against leishmaniasis by using a DNA plasmid containing the CMV promoter and encoding the major surface glycoprotein, gp63, from *Leishmania major* [48]. *Leishmania major* is responsible primarily for the cutaneous form of leishmaniasis; however, in some cases infection can lead to the visceral form of the disease. Immunisation of mice with this plasmid DNA encoding the gp63 induced T_H1 responses based on IL-2 and IFN-γ secretion from T cells obtained from the spleen and lymph nodes of immunised mice. No detectable IL-4 secretion

was observed when the splenocytes and lymph node cells obtained from gp63-DNA immunised mice were cultured with *L. major* antigens in vitro. The immunised mice also developed significant resistance against *L. major* infection when compared with control plasmid immunised mice. Other investigators have also reported the requirement for a T_H1 response to protect from cutaneous leishmaniasis, whereas a T_H2 response is not protective, and that DNA vaccines may be advantageous in this parasitic disease [49]. The T_H1 response to gp63 induced by DNA vaccines from *L. major* was correlated with protection against murine leishmaniasis [50]. The role of skin-derived dendritic cells as professional antigen presenting cells for priming the DNA induced T_H1 response has also been demonstrated. Protective immunity following DNA immunisation with the gene encoding the immunodominant LACK antigen from *L. major* was also reported in murine systems [51]. The control of disease progression and parasite burden in LACK DNA immunised mice was again associated with antigen-specific IFN-γ production and was dependent on IL-12, indicating the need for a T_H1 response. However, depletion of $CD8^+$ T cells at the time of LACK DNA immunisation or experimental infection also abolished protective immunity induced by LACK DNA injection. This suggested a role for $CD8^+$ T cells in this DNA vaccine induced protection to *L. major*. Together, these studies clearly demonstrate the potential of DNA immunisation for generating protective T_H1 responses in a parasitic system where T_H1 $CD4^+$ T cells are required for protective immunity.

Taenia is a genus of parasitic cestodes that infect the intestines of vertebrates, with *Taenia ovis* representing a tapeworm pathogen of sheep. DNA encoding a host protective antigen, designated 45W, from *T. ovis* was used to immunise sheep. DNA immunisation was compared with responses elicited by a recombinant 45W protein and an ovine adenovirus viral vector expressing the 45W antigen [52]. The effect of the route and mode of immunisation with DNA encoding 45W gene on the antibody response was also evaluated in sheep [53]. Sheep received two immunisations of the three different vaccination strategies. Low levels of specific 45W antibodies were induced by either DNA or the recombinant adenovirus vector immunisation [52]. The antibody levels were boosted by a subsequent immunisation with the recombinant 45W protein administered in Quil A as an adjuvant. The anti-45W levels after the recombinant protein boost were comparable with two injections of recombinant protein in adjuvanted. In the case of priming with DNA and/or

Table 1
Systems where DNA vaccines have been evaluated against parasites

Parasite	DNA vaccine	Reference
Plasmodium falciparum	Circumsporozoite protein antigen	[37]
	Multigene plasmid mixture	[39]
	Somatic transgene	[42]
Plasmodium berghei	Circumsporozoite protein antigen	[40]
	Pre-erythrocytic antigen	[41]
Schistosoma mansoni	28 kDa Glutathione *S*-transferase	[44,45]
	20.8 kDa Tegumental antigen	[46]
	Glutathione peroxidase	[47]
	Superoxide dismutase	[47]
Schistosoma japonicum	Paramyosin	[43]
	Paramyosin fused to 26 kDa glutathione *S*-transferase	[43]
Leishmania major	Surface glycoprotein, gp63	[48,50]
	Parasite surface antigen, Ag-2	[49]
	LACK protein antigen	[51]
Taenia ovis	Host protective antigen, 45W	[52]

the adenovirus vector and immunisation with the recombinant protein alone, sheep were protected from experimental challenge with *T. ovis*. These studies suggest that either DNA or recombinant adenovirus vectors can prime the immune response in sheep and the prime-boost strategy can elicit protective immunity. Systems where DNA vaccines have been evaluated against parasitic agents are summarised in Table 1.

7. Systems to develop DNA vaccines against animal coccidioses

A number of potential target antigens to develop DNA vaccination strategies against animal coccidioses is provided in Table 2. Studies that relate to the development and testing of DNA vaccination strategies against coccidia parasites are still in their infancy. A recently described study (S. Sugodira, D Buzoni-Gatel, S Iochmann, M Nacri, D Bout. Protection of neonates against cryptosporidiosis after genetic vaccination of dams. In: Proceedings Vaccines against Animal Coccidioses. COST 820, 1998; Annual Workshop, p 25) employed DNA immunisation to evaluate the vaccination potential against *Cryptosporidium parvum* in goats. *Cryptosporidium parvum* is an intracellular parasite that infects the epithelial lining of cells in the microvilli of the small intestine. This coccidian parasite infects humans and mammals causing cryptosporidiosis, which is an opportunistic infection of immunosuppressed individuals and is often associated with HIV infection and AIDS. Infection of humans is often caused by exposure to oocysts in water or the environment as the result of excretion by infected ruminants. The reduction of infection in the animal reservoir could have a direct impact on the rate of infection in humans. The intranasal genetic immunisation of mice with the gene encoding the *C. parvum* surface sporozoite CP15 antigen generates both humoral and cell-mediated immune responses. Pregnant goats were immunised three times with 200 μg of CP15 encoding plasmid DNA intranasally. The kids were subsequently infected 24 h after birth and monitored over a 3 week period. Nasal inoculation of pregnant goats with DNA encoding the *C. parvum* CP15 gene conferred protection against *C. parvum* in their infants. Kids from DNA vaccinated dams had a lower level of parasite development and their overall growth was not affected. This situation was different from that which was observed in the kids from the unvaccinated mother control groups where higher levels of parasites were obtained and the kids' growth was impeded. Other investigations have described serum and colostrum antibody responses in sheep following DNA injection with a plasmid encoding *C. parvum* CP15 and CP60 surface antigens [54]. The induction of immune responses in mice following DNA immunisation with plasmids encoding antigens from *Toxoplasma gondii* has also been described [55]. These studies provide evidence that DNA vaccination strategies can induce protective immunity in animal coccidioses systems.

Table 2
Potential target antigens to develop DNA vaccines against animal coccidioses

System	Putative antigen targets
Cryptosporidium parvum	Surface sporozoite, CP15 and CP60 antigens
	Sporozoite antigen, p23
Eimeria tenella	Microneme protein, Etmic 2
	Heat shock protein, HSP 70
	Merozoite surface antigens
Neospora caninum	Recombinant (rec) proteins, NC-p36 and NC-p43
Toxoplasma gondii	Surface antigens, SAG1 (p30), SAG3 (p43), and SAG4 (p18)
	Bradyzoite specific heat shock protein antigens
	Merozoite surface antigen, RAP6
Sarcocystis spp.	Surface sporocyst antigens

8. Human clinical trials with DNA vaccines

There are presently a number of ongoing clinical human trials that are evaluating the effectiveness of DNA vaccination for both prophylaxis and therapy. These trials include HIV, HSV, influenza, malaria, and cancers (Table 3). The

Table 3
DNA vaccination strategies in human clinical trials

Organism/system	Plasmid DNA employed	Utilisation
HIV-1	HIV-1 gp160	Therapeutic
HIV-1	HIV-1 gag/pol	Therapeutic
HIV-1	HIV-1 gp160	Prophylactic
Malaria	Malaria CSP	Prophylactic
HSV-2	HSV-2 gD	Therapeutic
Influenza	Influenza NP	Prophylactic
Hepatitis B	Hepatitis B surface antigen	Prophylactic
Cutaneous T cell leukemia	TCR idiotype	Therapeutic
Carcinoma	CEA	Therapeutic

first DNA based vaccine trial was reported for therapy of HIV infection [56]. A DNA construct expressing the *env* and *rev* genes of HIV-1 was used to immunise 15 asymptomatic HIV infected individuals who were not using antiviral drugs and who had normal levels of $CD4^+$ lymphocytes. Treatment groups received three doses of vaccine (30, 100, 300 μg) at 10 week intervals in a dose-escalation trial. The DNA vaccine was safe and well-tolerated. Antibody responses to HIV gp120 increased among individuals given the higher doses of DNA. Additionally, some increase in CTL activity against gp160 expressing target cells and lymphoproliferative activity was observed.

More recently, a human clinical trial examined a *Plasmodium* derived DNA prophylaxis [57]. Twenty healthy, malaria naive individuals were randomised into four groups of five individuals, and given three injections of 20, 100, 500, and 2500 μg of plasmid DNA encoding the *P. falciparum* CSP at 4 week intervals. These individuals elicited antigen-specific, genetically restricted $CD8^+$ CTL responses as the result of DNA vaccination. This represented the first study where healthy naive humans generated $CD8^+$ CTL responses as the result of DNA immunisation. These two studies are the first published reports describing human clinical trials that evaluated DNA immunisation and they demonstrate that DNA immunisation is capable of generating and boosting the immune response to specific antigens associated with human pathogenic organisms.

9. Issues related to DNA vaccines

DNA vaccines afford a number of advantages and possible disadvantages when compared with alternative vaccination strategies (reviewed in [58] and [59]). The advantages of DNA vaccines include the fact that they can encode multiple immunogenic epitopes and evoke both humoral and cell-mediated immune responses. The immunogenic epitopes are presented to the immune system in their native form. Thus, DNA exhibits the advantages of attenuated vaccines without the safety problems associated with in vivo replication and possible reversion to a virulent form. Plasmid vectors can be rapidly constructed and easily tested. Large-scale manufacturing procedures are available and the DNA can be easily and inexpensively purified to homogeneity, resulting in lower costs to develop and manufacture this type of vaccine. This makes this strategy applicable as a human vaccine approach in underdeveloped countries and as a veterinary vaccine strategy where the cost per dose is of major economic concern. DNA is more thermostable than vaccine strategies which require a cold chain for storage. It should exhibit a longer shelf-life because of the improved stability. The production of combination vaccines employing DNA is also simplified. DNA also allows a more simplified and effective quality control process that provides additional cost benefits.

Some concerns and potential disadvantages of DNA vaccines also exist. These include the potential for integration of the DNA into the host chromosome. Plasmid DNA based vectors often contain nucleic acid sequences from oncogenic viruses, and the possibility for chromosomal integration exists. A second concern of DNA vaccination is the possibility of generating antibodies to DNA. Immune responses to DNA occur in autoimmune diseases, such as systemic lupus erythematosus, and the potential exists that bacterial DNA injection could induce an immune response that might cross-react with host DNA. It

has been reported that antibodies to DNA have been observed following immunisation of mice with bacterial DNA [59,60]. However, this still represents a theoretical possibility that will require more attention. A third concern is the effect that long-term expression of injected DNA into muscle cells may have on immune responses to subsequent vaccination with different DNA, and whether the immune responses to protective epitopes associated with this second immunisation will be compromised. The unwarranted effects of "original antigenic sin" may come into play in this scenario. The fourth disadvantage is that DNA vaccination strategies are unsuccessful when evaluating non-protein based antigens, such as bacterial polysaccharides and lipids. Yet DNA immunisation represents a promising and new approach to vaccine development that is worthy of evaluation. It remains to be determined how successful DNA vaccines will ultimately become in the future, and how widespread their prophylactic and therapeutic applications will be in the areas of infectious diseases and cancer. Based on present studies in human clinical trials and the multiple successes in a variety of animal models, it appears that DNA immunisation will not fall by the wayside of other vaccination strategies that failed to live up to their initial promise touted during the basic research phase of their evaluation.

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